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THE
BIOLOGICAL BULLETIN

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# NATURAL HETEROAGGLUTININS IN THE SERUM OF THE SPINY LOBSTER, PANULIRUS INTERRUPTUS. II. CHEMICAL AND ANTIGENIC RELATION TO BLOOD PROTEINS[1]

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In a previous report (Tyler and Metz, 1945) it has been shown that lobster-serum contains at least ten heteroagglutinins for sperm or blood cells of various animals. Each of the heteroagglutinins was found to act on all the species tested that belong to the same group of animals. Since the group, in most instances, represents a taxonomic class, the heteroagglutinins are termed class-specific. The heteroagglutinins were found to be most probably protein, and by means of electrophoresis they were shown to be distinct from the hemocyanin which Allison and Cole (1940) and Clark and Burnet (1942) had considered to be the sole protein present in lobster-serum.

The relatively small amount found to be present accounts for Allison and Cole's conclusion which was based on approximate identity of the copper to protein nitrogen ratios of purified hemocyanin and of whole serum. Clark and Burnet's evidence was actually to the effect that there is no protein present with active antigenic properties different from that of pure hemocyanin. This is in accord with the results obtained with antisera prepared against heteroagglutinin by injecting rabbits with agglutinin that had been absorbed on rabbit cells. In the present paper a precipitation method for preparing the heteroagglutinins free of hemocyanin is described, and results of an electrophoretic examination of the material are presented. The agglutinating action of fibrinogen preparations from plasma and further serological tests are also reported.

## Material and Methods

Blood is quite easily obtainable from lobsters by means of a syringe inserted, between cephalothorax and abdomen, into the pericardial chamber. A twelve-inch lobster yields, in this manner, about 20 to 30 ml. of blood. For serum the blood was generally defibrinated by shaking with glass beads, filtered, and centrifuged; or it was occasionally allowed to clot, forced through a fine mesh wire screen, and centrifuged. For plasma the blood was drawn into a small amount of sodium

[1] This work has been aided by grants from the American Philosophical Society and the Rockefeller Foundation.

citrate solution, then subsequently filtered, centrifuged, and dialyzed against saline. One volume of 10 per cent citrate suffices to prevent fibrin-clotting in about 30 volumes of blood.

The agglutinative tests were made as previously described (Tyler and Metz, 1945) by mixing equal volumes of the sperm or blood cells (of sea-urchin, sheep, or other animal) and of serial two-fold dilutions of the test-solution adjusted to the appropriate salinity. Deviations from these proportions are specified in the tests.

## Experimental Part

### *Separation of heteroagglutinins from hemocyanin by isoelectric precipitation*

Hemocyanin was prepared from serum by isoelectric precipitation essentially as described by Allison and Cole (1940) and by Rawlinson (1940). This consists in dialysis against distilled water and then against dilute acetate buffer at the pH of the isoelectric point. Further purification is obtained by repeated solution in dilute ammonia and reprecipitation, by addition of acetate buffer (0.1 M., pH 4.5).

Rawlinson (1940), in the course of purification of hemocyanin from the *plasma* of the Australian spiny lobster, noted the presence of small amounts of protein which he considered to be fibrinogen. Such a non-hemocyanin protein is obtainable from the *serum* of the California spiny lobster, *Panulirus interruptus*.

When samples of serum or plasma of *Panulirus* were dialyzed against dilute, pH 4.5, acetate buffer, there invariably appeared small amounts of a pale precipitate that separated before the hemocyanin started to come down. The precipitates ranged in color from white to pink. After centrifugation, washing with distilled water and solution in dilute ammonia, the material was reprecipitated by slow addition of 0.01 M., pH 4.5 acetate buffer. The material was regularly found to start to precipitate at pH 5.0 and reach a maximum at pH 4.8. From the supernatants of the first precipitates the blue-colored hemocyanin was precipitated by continuation of the dialysis against the pH 4.5 buffer. The hemocyanin was obtained in crystalline form from concentrated solutions of it in dilute ammonia by the slow addition of dilute acetate buffer. Its precipitation was found to begin at pH 4.6 and to be complete at 4.5 to 4.4.

Samples of the purified hemocyanin and of the pale precipitate were tested for their ability to agglutinate the sperm or blood cells of various animals. After adjustment of the solution to appropriate pH and salinity by dialysis, they were tested on one per cent suspensions of the sperm of the polychaet, *Chaetopterus variopedatus;* the sea cucumber, *Stichopus californicus;* the starfish, *Pisaster ochraceus*; the sea-urchin, *Strongylocentrotus purpuratus*; the sea-squirt, *Ciona intestinalis;* and the grunion (smelt), *Leuristhes tenuis;* and of the erythrocytes of the sand bass, *Paralabrax maculatofasciatus;* the frog, *Rana pipiens;* the chuckwalla, *Sauromalus ater;* the pigeon, and sheep. The hemocyanin preparations, containing this material in amounts as great as or greater than normally present in the serum, were found to be completely inactive. The preparations of the pale, pH 4.8-5.0, precipitate gave very good agglutination of the cells of all the above named species.

Titer determinations were made with one of these preparations on sperm of *Strongylocentrotus*. In this case 0.2 ml. of serial two-fold dilutions of the solution were mixed with one drop of 10 per cent sperm-suspension. The protein con-

centration (from Kjeldahl nitrogen determination) of the solution was 0.7 per cent and its titer (minimum dilution giving definite microscopic agglutination) was 128. A sample of serum containing 5 per cent protein gave at the same time a titer of 256. This preparation showed, then, about 3½ times the activity of the whole serum.

*Electrophoretic examination of the pale precipitate* [2]

Another sample of the material freed of hemocyanin was reprecipitated at pH 5, dissolved in dilute ammonia, and dialyzed for 2 days in the cold against barbiturate buffer ($\mu = 0.05$) at pH 7.7. It was then examined electrophoretically in the

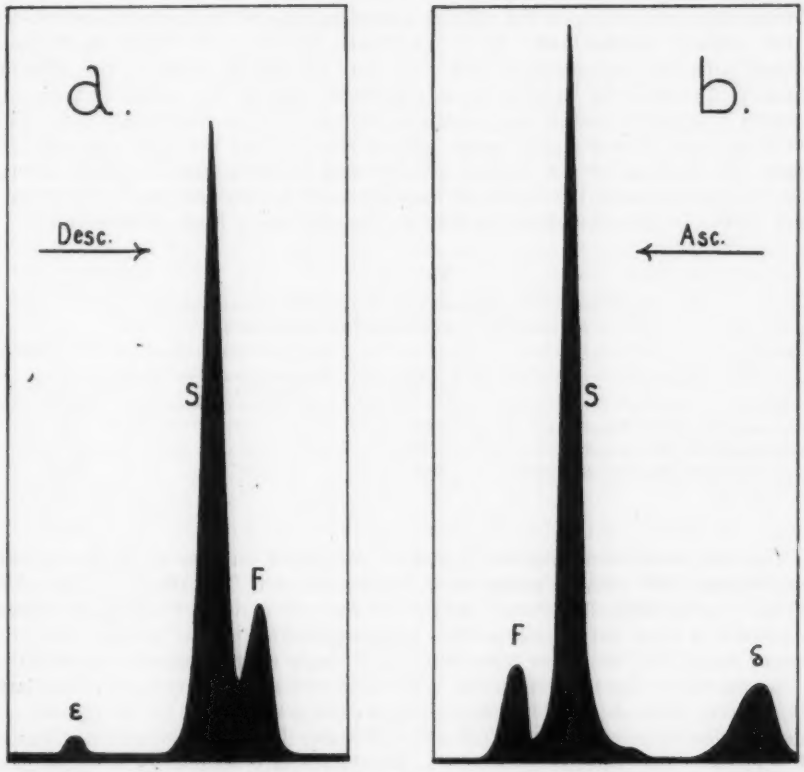


FIGURE 1. Electrophoretic patterns of pale (pH 5) precipitate from lobster-serum. *a*, descending (desc.) side; b, ascending (asc.) side; after 59 minutes of electrophoresis at pH 7.7, ionic strength 0.05 and 14.8 ma. Arrows show direction of migration. See text for further description.

[2] The apparatus employed was that constructed in the Division of Chemistry by Dr. Stanley M. Swingle to whom we are indebted for the electrophoresis of this material.

Tiselius' (1937) apparatus using the scanning method of Longsworth (1939). After 59 minutes of electrophoresis with a current of 14.8 ma., the patterns shown in Figure 1 were obtained. As may be seen from the figure two components, besides the δ- or ε-boundaries, are present in the serum. From the relative areas covered by the peaks the ratio of amount of slow component to that of fast component is approximately 5:1. At the end of the run the fast moving component was removed from the ascending side and the slow component (plus δ), from the descending side of the electrophoresis cell. After dialysis against normal saline, determinations were made of their agglutinative titers for rabbit cells and of the Kjeldahl nitrogen content. Samples of the original solution of the pale precipitate (taken from the cell after the run) and of normal lobster-serum were tested at the same time. The results are given in Table I. The nitrogen content of the solutions does not represent the relative concentrations of the components present in the original solution since there was some dilution with buffer upon their removal from the electrophoresis cell. As may be seen in Table I, the solution of the fast component showed no agglutinative activity for rabbit-erythrocytes although its nitrogen content was about one-third that of the slow component. The slow component proved highly active, giving almost twice the titer (per mg. N. content of solution) of the original solution and 24 times that of whole serum. This is approximately the order of magnitude of activity obtained (Tyler and Metz, 1945) for the components isolated by electrophoresis from whole serum.

TABLE I

*Agglutinative titers of components obtained by electrophoresis of the pale (pH 5) precipitate from lobster-serum*

| Material | mg. Kjeldahl N. per ml. | Agglutinative titer on 1% rabbit cells | Titer/mg. N. |
|---|---|---|---|
| Fast component (F of Figure 1) | 0.29 | 0 | 0 |
| Slow component (S of Figure 1) | 1.008 | 128 | 128 |
| Original solution (from cell-residue) | 3.85 | 256 | 66.5 |
| Whole serum | 11.97 | 64 | 5.3 |

The slow component obtained here was also tested on cells of all the animals listed on page 194, with the exception of *Sauromalus* and *Leuristhes*. It proved to be highly active with all of them. In the previous report lobster-serum was shown to contain at least ten "class-specific" heteroagglutinins. It is evident from the present results that these are represented by a single electrophoretic component of the serum, unless there is some active component in the stationary δ- or ε-boundary. The latter is, however, highly unlikely since the original material for the present test was obtained by precipitation at pH 4.8 to 5.0 and the electrophoresis was run at pH 7.7. For any material to remain in these stationary boundaries it would have to be isoelectric at the latter pH.

*Preparation of fibrinogen and tests for heteroagglutinating activity*

Lobster-plasma upon being brought to 25 per cent saturation with ammonium sulfate formed a white to pink precipitate which separated easily upon centrifuga-

tion. The precipitate was washed with distilled water and dissolved in sea water. Addition of fresh lobster-blood-cells to the solution caused it to form a firm clot. A pH 5.0 precipitate obtained directly from plasma was found to contain fibrinogen, which could be separated from the remaining protein material by precipitation with ammonium sulfate. None of the prepartions from serum were found to contain fibrinogen.

TABLE II

*Agglutinative titers of protein preparations from plasma and serum*

| Material | mg. Kjeldahl N. per ml. | Agglutinative titer on *Strongylocentrotus* sperm | Titer per mg. N. |
|---|---|---|---|
| Fibrinogen preparation (I) | 1.25 | 32 to 64 | 26 to 51 |
| Pale precipitate (II) | 1.25 | 64 to 128 | 51 to 102 |
| Hemocyanin | 7.4 | 0 | 0 |
| Whole serum | 8.5 | 128 to 256 | 15 to 30 |
| Plasma | 8.5 | 256 to 512 | 30 to 60 |

A fibrinogen preparation (I) was obtained from whole plasma by 25 per cent saturation with ammonium sulfate. The precipitate was dissolved and reprecipitated by dialysis to pH 5.0. This preparation was tested for agglutinating action on sperm of *Strongylocentrotus* in the same manner as on page 194. The supernatant from the 25 per cent ammonium sulfate precipitate was dialyzed against tap water and then brought to approximately pH 5 by dialysis against pH 4.5 buffer. This gave a pale precipitate (II) which resembled the pale precipitate from serum. After solution and dialysis against sea water it, too, was tested for agglutinating activity. The results are given in Table II along with simultaneous tests of whole serum, plasma, and hemocyanin. The presence of calcium in the sperm suspension does not interfere with the tests, since clotting of the fibrinogen does not occur unless fresh lobster-blood-cells are added. As the table shows, plasma has about twice the agglutinating activity of serum. The fibrinogen preparation proved about half as active as the pale precipitate.

Another pale precipitate was also obtained directly from plasma by dialysis against pH 4.5 buffer. When the precipitate was dissolved and brought to 25 per cent saturation with ammonium sulfate there separated out some material that proved to be fibrinogen. It appears from the experiments reported above that the isoelectric point of fibrinogen is not greatly different from that of the heteroagglutinin found in serum. This conclusion was verified by Mr. Maurice Rapport, who repeated some of our experiments, and made an electrophoretic examination of plasma and of protein preparations separated from plasma. The pH 5.0 precipitate from plasma showed two electrophoretic components, the patterns being similar to those of Figure 1. The smaller, faster component probably corresponded to the fast component observed in serum preparations. The other component, containing agglutinating activity, could not be separated further during 100 minutes of electrophoresis at pH 7.3, 1.2° C. and 20 ma.

Precipitation of the pH 5.0 precipitate from plasma with ammonium sulfate at 40 per cent of saturation removed nearly all of the agglutinating activity, but left behind a small amount of protein material. The ammonium sulfate precipitate

contained 3.5 mg. Kjeldahl N./ml, and had a titer of 64 against *Strongylocentrotus* sperm. The supernatant contained 1.6 mg. N./ml, and titrated only to 4. Mr. Rapport showed that this small residue migrated rapidly in the electrophoresis apparatus at pH 7.3. It probably corresponded to the fast component from serum.

In the absence of more exhaustive chemical and electrophoretic separations it is not possible to decide with certainty whether the agglutinative activity found in fibrinogen preparations is associated with fibrinogen itself, or is due to the presence in these preparations of the heteroagglutinin fraction which is present in serum.

*Antigenic relationship of the blood proteins*

Two rabbits that were each given two courses of intravenous and intra-abdominal injections with a total of 375 mg. of purified hemocyanin produced very good precipitating antisera. The titers (end point of precipitation on mixing equal volumes of antiserum and serial dilutions of a 10 per cent hemocyanin solution) ranged from 10,000 to 20,000 in terms of antigen dilution and optimal proportions (second optimum, see below) were obtained at approximately one volume of 10 per cent hemocyanin to 10 to 20 volumes of antiserum. The antisera also reacted very well with whole lobster-serum, the optimal proportions point being about 9 volumes of antiserum to one volume of the lobster-serum.

Tests were then made of the ability of antiserum vs. hemocyanin to remove natural heteroagglutinin from whole lobster-serum. One volume of lobster-serum was absorbed with 9 volumes of the rabbit antiserum and the supernatant tested for ability to agglutinate rabbit-erythrocytes and *Strongylocentrotus* sperm. The absorbed serum gave no reaction with these cells, while control lobster-serum gave good agglutination out to dilutions of 1/90 (+ + + reaction) with the rabbit cells and 1/80 (+ reaction) with the *Strongylocentrotus* cells respectively.

It appears, then, that antibodies prepared against hemocyanin also react with the natural heteroagglutinins present in lobster-serum.

One of the antihemocyanin rabbit sera was also titrated with the solution of electrophoretically purified heteroagglutinin (slow component). A titer (dilution of antigen) of 128 was obtained for this solution which contained one mg. Kjeldahl N. per ml. A control hemocyanin solution containing 8 mg. N. gave a minimum titer (end point not reached) of 4096, or 512 per mg. N.

Another antiserum against hemocyanin was also titrated with various protein fractions separated from lobster-blood. The titer (dilution of antigen) of reprecipitated hemocyanin was 20,000 for a solution containing 6.6 mg. Kjeldahl N. per ml. or 3000 per mg. N. For the heteroagglutinin (pH 5 precipitate from serum, reprecipitated), the titer was 200 for a solution containing 1.6 mg. N. per ml. or 125 per mg. N. For the fibrinogen (ammonium sulfate precipitate from plasma), the titer was 200 for 3.4 mg. N. per ml. or 60 per mg. N.

In these titrations, it was sometimes noted that precipitation occurred in the first few tubes, containing concentrated antigen solutions. In intermediate dilutions, no precipitation occurred, but a second zone of precipitation appeared in the higher dilutions. This was noted both with hemocyanin and fibrinogen, but not with the agglutinin preparation (pale precipitate from serum) used. Boyden and deFalco (1943) reported a similar double zone phenomenon with *Homarus* serum titrated against anti-*Homarus*-hemocyanin. They pointed out that this is indicative of the

presence of two kinds of antibodies in the antisera. However, this does not seem to be the entire explanation, since we find that absorption of a sample of antiserum with an amount of hemocyanin which corresponds to the lower of the two optima removes all antibody for the homologous antigen, as well as for fibrinogen and pale precipitate.

Two rabbits were also immunized with whole lobster-serum, each receiving a total of 5.5 ml. of serum in two courses of three injections each, with three weeks rest between courses. The antisera obtained one week after the last injection gave very good precipitation with the homologous antigen, optimal proportions (second optimum) being obtained with one volume of lobster-serum to approximately 16 volumes of antiserum. A sample was absorbed with purified hemocyanin and tested on whole serum, a concentrated solution of the pale (pH 5) precipitate, and a fibrinogen preparation. It failed to give precipitation with any of these antigens. This confirms the findings of Clark and Burnet (1942) and indicates that the other blood proteins have no active antigenic groups other than those present in the hemocyanin. Alternatively, the results might be explained on the basis of competition of antigens (see Sachs, 1929), such that the rabbit does not form antibodies against other antigens when one powerful antigen (the hemocyanin) is present in excess in the material (whole lobster-serum) used for immunization. However, in view of the analogous results obtained (Tyler and Metz, 1945) with antisera prepared against heteroagglutinin, and with antihemocyanin sera (above), the alternate explanation seems highly unlikely.

## Summary

1. Lobster-serum contains small amounts of other protein constituents besides hemocyanin.

2. The "class-specific" heteroagglutinins of lobster-serum are found to reside in a component that is obtained free of hemocyanin by isoelectric precipitation at pH 4.8 to 5.0.

3. Electrophoresis of this "pale precipitate" reveals the presence of two components, of which the more slowly migrating one bears the heteroagglutinating activity. The ten separate "class-specific" heteroagglutinins are thus evidently represented by a single electrophoretic component.

4. There is some indication that fibrinogen obtained from the lobster plasma may also act as heteroagglutinin.

5. Antibodies produced in rabbits against purified hemocyanin also react with the slow electrophoretic component (heteroagglutinin) of the pale precipitate and with fibrinogen. Absorption tests with antisera vs. whole lobster-serum fail to reveal the presence of any specific antigenic groups other than those of the hemocyanin. The other blood proteins are, then, evidently serologically equivalent to hemocyanin.

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# STUDIES ON MARINE BRYOZOA. I. AEVERRILLIA SETIGERA (HINCKS) 1887

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## Introduction

During the summer of 1944 collections of *Aeverrillia setigera* were made at New Bedford and Woods Hole, Massachusetts. Perusal of literature pertaining to this species showed that a more complete account of this form would not be amiss. This article brings together all available distribution and anatomical data previously given for this form and adds to it some new distribution data, more complete illustrations than were heretofore available and a considerable amount of anatomical and some ecological data.

The writer wishes to acknowledge, with sincere appreciation, the kindness of Dr. Hannah Croasdale of Dartmouth College and of the Marine Biological Laboratory of Woods Hole, Mass., who collected the first specimens of *A. setigera* from New Bedford, Mass., and turned them over to the writer for study, and to Dr. Raymond C. Osburn of the University of Southern California who so kindly checked the specimens, confirming the identification and who offered many helpful suggestions.

## Distribution

The species *Buskia setigera* has been reported previously by the following authorities from the localities listed below:

Hincks, 1887 (pp. 121, 127-128; Pl. XII, Figs. 9-13), from the Gulf of Bengal, around the Mergui Archipelago.

Kirkpatrick, 1890a (pp. 603, 612), between Australia and New Guinea in the Torres Straits, 20 miles off Warrior Island.

Kirkpatrick, 1890b (p. 17), off Tizard Banks in the China Sea.

Thornely, 1905 (p. 128), from Ceylon.

Harmer, 1915 (pp. 87-88; Pl. 5, Figs. 8-10), from the Bay of Bima (India), Bay of Badjo, west coast of Flores (Malay Archipelago), Makassar, Borneo Bank, off Pulu Jedan, east coast of Aru Islands, and also in the following unnamed locations: Station 164, at 1°42′.5 S.; 130°47′.5 E.; Station 166, at 2°28′.5 S.; 131°3′.3 E.

Thornely, 1916, off Poshetra Head, Kattiawar, and Ceylon.

Hastings, 1927 (p. 351), at Menzaleh Lock and other stations at the Suez Canal.

Livingstone, 1927 (p. 67), from Queensland, Australia.

Hastings, 1932 (p. 407), from Penguin Channel and N. E. Low Island, Great Barrier Reef, Australia.

Osburn, 1933 (p. 64), from Porto Rico.

Marcus, 1937 (p. 143; Pl. 29, Fig. 76), from Bay of Santos, Brazil, South America.

Osburn, 1940 (p. 343), from Porto Rico.

Hutchins, 1945 (p. 539), Pine Orchard, Long Island Sound, Connecticut, U.S.A.

Additional discussion of the species occurs in the following articles:

Osburn and Veth, 1922; (p. 159).

Marcus, 1938; (p. 61).

Marcus, 1939; (pp. 168, 171).

Marcus, 1941; (pp. 74-77, 147; Pl. X, Fig. 45).

The above reports indicate that the species is distributed near several continents, —Africa (Suez Canal), Asia, Australia, South America, and North America, and also around several islands, including Porto Rico. The present article reports its occurrence around the State of Massachusetts, extending the northerly range of this species to 41°38′ N. Latitude.

*Averrillia setigera* was found in two Massachusetts localities. The first collection was made by Dr. Hannah Croasdale on July 29, 1944, at Black Rock in the Harbor of New Bedford, Mass. The next collections were made by the author on August 4, 13, and 14, 1944, at Stony Beach, Woods Hole, Mass. Further details of the nature of the collecting site and the associated biota will be given in the ECOLOGY section.

## Ecology

The New Bedford Harbor specimens were collected by Dr. Croasdale at the time of low tide, from the littorine region around Black Rock, along with red algae, at a depth of less than 2 feet below the surface of the water. The Woods Hole specimens came from a large, partially submerged boulder located approximately 50 yards from shore. The sea bottom around the boulder is largely sand although there are some rocks a short distance away on each side of the boulder. The general locality is not subjected to strong wave action. The boulder is almost completely submerged at high tide but is about half exposed at low tide. Its sides are well covered with algae of various kinds as well as with a luxuriant fauna. The *A. setigera* colonies were collected at low tide, a foot or two below water level, by gathering likely looking *Chondrus* and *Ascophyllum* algae off the boulder.

The Woods Hole *A. setigera* specimens were found growing in close association with the following animal forms: *Folliculina, Vorticella, Sycon, Obelia, Sertularia,* other hydroids, *Bowerbankia gracilis, Bugula flabellata, Crisia eburnea,*

*Hippothoa hyalina, Pedicellina cernua,* and *Stephanosella biaperta.* The *Aeverrillia* autozoids, and in some instances stolons, had a few *Folliculina, Vorticella,* or *Pedicellina,* growing on them. The *A. setigera* colonies grew on hydroid stems and on the same algal thalli (*Chondrus* and *Ascophyllum*) as *Bugula flabellata, Hippothoa hyalina, Crisia,* and the other Bryozoa.

*Aeverrillia setigera* has been collected from varying depths, from one or two feet below tide mark (present author) to much greater depths (other writers). Kirkpatrick found specimens at depths of 5½ and 27 fathoms; Thornely (1916), at 7 fathoms; Hastings (1932), at 8 to 15½ fathoms; Marcus (1937), at 17 meters; while Harmer found specimens at greater depths: 0 to 40 meters, 55 meters, 59 meters and 118 meters.

This Bryozoan grows on the following types of substratum: 1, on broken shells (Kirkpatrick, 1890a); 2, on stems of *Idia pristis* (Thornely, 1916); 3, on stems of hydroids and Bryozoa (Osburn, 1940); 4, on stems of *Nellia oculata* Busk (Hincks, 1887); 5, on hydroids and the following Bryozoa: *Bugula, Catenicella* and *Valkeria atlantica,* which were dredged from areas whose bottom consisted of such materials as mud, sand, hard coarse sand, coral, shells, and stones (Harmer, 1915); and 6, on hydroids like *Obelia* and algae like *Chondrus* and *Ascophyllum,* in close association with many other already mentioned animal forms (present paper).

## Description of Species

The status of Bryozoa as an entire group is still an unsettled problem. It has been considered a Phylum, a Sub-phylum and a Class. Each category has its earnest and qualified supporters. With this in mind the following taxonomy of the *Aeverrillia* species, patterned after the work of Dr. Marcus, is given:

——— BRYOZOA Ehrenberg 1831
Class ECTOPROCTA Nitsche 1869
Order GYMNOLAEMATA Allman 1856
Sub-order CTENOSTOMATA Busk 1852
Group STOLONIFERA Ehlers 1876
Family Valkeriidae or Mimosellidae?
Genus *Aeverrillia* Marcus 1941
Species *setigera* Hincks 1887

The classification of *Aeverrillia setigera* has undergone a few changes since its original description by Hincks in 1887. Its generic names were *Buskia, Hippuraria,* and now *Aeverrillia.* The latter genus was erected in 1941 by Dr. Marcus in honor of A. E. Verrill.

The question regarding the family into which it should be placed is set forth by Marcus (1941, p. 147) thus: "*Aeverrillia* does not need a new family; the genus can be placed in the Valkeriidae or perhaps in the Mimosellidae as now enlarged by Bassler (1935, p. 8)." Earlier the species had been placed among the Triticellidae, the Buskiidae, and eventually into the Valkeriidae.

The colonies are delicate yellowish or very pale amber colored transparent traceries closely adherent to various living and non-living submerged objects. They are barely big enough to be seen with the unaided eye. They consist of paired

individuals connected by slender stolons. The stolons and individuals are chitinized and firm-walled. The stolons especially have a well thickened wall.

Bryozoa exhibit polymorphism. The *Aeverrillia* colony consists of three types of structures or possibly individuals, namely stolons, peduncles, and autozoids.

In the colony there is a main or primary axis or stolon and lesser (secondary and sometimes tertiary) stolons (Fig. 7). The lesser stolons are more apparent in older colonies than in young ones.

These stolons, according to Dr. Marcus, are composed of kenozooecia. The long slender tubular kenozooecia of each stolon grow longitudinally and are attached end to end. Those of the secondary stolons have their origin at the sides of the primary stolon usually with a peduncle intervening between the primary and secondary stolons. The tertiaries have their origin at the sides of the secondaries, likewise usually with an intervening peduncle. Some stolons appear to arise directly from other stolons without an intervening peduncle (Fig. 8). Also, one of a pair of opposite stolons may arise from a stolon without the intervention of a peduncle while its partner may have a peduncle between it and its parent stolon (Fig. 8). Whether this barrenness of one stolon may be a temporary or a permanent condition is uncertain.

The primary and secondary stolons are at right angles, approximately, to each other but it is difficult to say the same about the tertiaries because the latter are sometimes twisted, gnarled, and not often found running in a straight line because of the limited area of the substratum on which the specimens grow. The secondary stolons usually originate in pairs, one stolon on each side of the primary stolon and directly opposite the other, growing away from each other.

The primary stolons, possibly because they are older, have thicker walls than the secondary stolons. The primaries are also somewhat straighter than the secondary and tertiary stolons but that again may be due to the limited substratum. Anastomoses occur occasionally, especially where there are tertiary and secondary stolons over a crowded or limited substratum. Hincks suggested the possibility of anastomosis of branches.

Primary stolons are very closely and entirely adherent to the substratum which in many cases proves to be a hydroid stem or *Chondrus* or *Ascophyllum* thallus. The primaries follow the stems or thalli in a fairly straight line for some distance. The secondaries and tertiaries must find what surface they can. Some of the lesser stolons look as if they are not necessarily attached along their entire length.

Generally the kenozooecia of the stolons are slightly enlarged distally at the point of origin of the lateral kenozooecia or peduncles. Transverse uniporous septa mark the proximal and distal limits of the kenozooecia along the stolons (Figs. 2 and 10). There are septa also at the points of origin of the lateral branches on the main stolons (Figs. 16 and 18). The region of the septum is sometimes referred to as the node and the stretch of stolon between two transverse septa, as the internode.

Stolon length is variable (Figs. 7 and 9, Table I). Some secondary stolons are short, some long. Some tertiary stolons are considerably longer than the primaries or than some of the secondaries. Stolon diameter is given in Table I.

The stolons under low power observation (75 × magnification) appear empty or tubular but under higher magnification (430 ×) a cellular lining membrane is evident within them.

TABLE I

*Measurements of Massachusetts specimens of Aeverrillia setigera*

| Part | Number of readings | Maximum | Minimum | Average | Refer to Figs. |
|---|---|---|---|---|---|
| A. Length or height of furled setigerous collar | 17 | 0.602 mm. | 0.440 mm. | 0.531 mm. | 6, 17 |
| B. Diameter at distal end of unfurled setigerous collar | 5 | 0.537 mm. | 0.370 mm. | 0.440 mm. | 6 |
| C. Diameter at the basal, proximal end of the setigerous collar | 6 | 0.110 mm. | 0.059 mm. | 0.083 mm. | 6, 17 |
| D. Length of orificial spine | 20 | 0.259 mm. | 0.141 mm. | 0.204 mm. | 6 |
| E. Diameter of extruded vestibular membrane | 1 | | | 0.111 mm. | 6 |
| F. Length of extruded vestibular membrane | 1 | | | 0.321 mm. | 6 |
| G. Length of tentacular sheath | 1 | | | 0.237 mm. | 6 |
| H. Diameter of tentacular sheath | 2 | 0.096 mm. | 0.074 mm. | 0.085 mm. | 6, 11 |
| I. Autozoid width at widest part | 7 | 0.212 mm. | 0.170 mm. | 0.185 mm. | 8 |
| J. Autozoid length from base of zoid to base of orificial spines | 18 | 0.592 mm. | 0.481 mm. | 0.552 mm. | 8 |
| K. Stolon diameter, at the normal thickness, not the swollen area of the stolon | 24 | 0.049 mm. | 0.015 mm. | 0.027 mm. | 8 |
| La. Length of shorter lateral surface of the clasping processes | 23 | 0.179 mm. | 0.043 mm. | 0.110 mm. | 7 |
| Lb. Length of longer lateral surface of the clasping processes | 23 | 0.182 mm. | 0.077 mm. | 0.119 mm. | 7 |
| M. Width of stolon at most swollen part, near node | 18 | 0.051 mm. | 0.034 mm. | 0.040 mm. | 8 |
| N. Length of peduncle | 19 | 0.170 mm. | 0.068 mm. | 0.114 mm. | 18 |
| O. Diameter of peduncle | 19 | 0.071 mm. | 0.039 mm. | 0.058 mm. | 8 |
| P. Length of internode | 24 | 1.013 mm. | 0.294 mm. | 0.658 mm. | 9 |
| S. Number of tentacles | 6 | 8 | 8 | 8 | 6, 13, 14 |
| T. Number of setae in setigerous collar | 3 | 19 | 16 | 17 | 6 |

## PLATE I

All figures except Figures 2 and 6 have been drawn with the aid of a camera lucida. All are of *Aeverrillia setigera.*

FIGURE 1. A chitinized sconce of the proventriculus, seen from the lumen side. Note the converging rows of teeth. Drawn to the same scale as Figure 10. This and Figures 3, 4, and 5 are from gizzard remains found in empty, degenerated autozooecia.

FIGURE 2. Detail of the uniporous septum which occurs along the stolons.

FIGURE 3. Latero-basal view, from the concave side of the chitinized gizzard sconce. All the softer parts of the gizzard have disintegrated, leaving only the hardened plate or sconce. Drawn to the same scale as Figure 10.

FIGURE 4. Side view of a somewhat flattened chitinized gizzard sconce. Drawn to the same scale as Figure 10.

FIGURE 5. Side view of a chitinized gizzard sconce of the more usual shape. Some of the teeth are darker than others. Drawn to the same scale as Figure 10.

FIGURE 6. A diagram showing several things: the relation between the open or unfurled setigerous collar, the eight tentacles, three of the four zooecial spines around the orifice and the lettered areas A through G along which measurements for Table I have been made. The same letters are found listed in the first column of the Table.

Line A stands for the length or height of the setigerous collar. It was measured only when furled or very slightly unfurled.

Line B represents the diameter of an unfurled setigerous collar, at its distal, expanded end.

Line C represents the diameter at the basal or proximal end of the setigerous collar.

Line D represents the length of the orificial spine.

Line E represents the diameter of the vestibular membrane and the area it encloses.

Line F represents the length of the vestibular membrane.

Line G represents the length of the tentacular sheath or the distance between the lophophore and the base of the setigerous collar, C——C.

FIGURE 7. Part of an old zoarium or colony showing the growth habit, anastomosis of branches (AN), primary stolon (P.ST.) and secondary stolon (S.ST.). All the autozooecia are empty of polypides. One (Z) has the setigerous collar in place yet but has no polypide. Some of the zooecia have two or three acuminate processes (B.P.). Measurements of these acuminate processes were made along two surfaces, the shorter (La) and the longer (Lb). These figures are to be found in Table I. The "membranous" area mentioned by Hincks is not very plain on most specimens. However, there is a hint of it in the second and fourth zooecia from the top. Drawn from freshly collected material on Aug. 14, 1944, and to the scale shown at its base.

FIGURE 8. An autozooecium attached to a very long secondary stolon. The location of certain measurements mentioned in Table I is indicated on the drawing.

Line I represents the diameter of the autozoid.

Line J represents the length or height of the autozoid exclusive of spines.

Line K represents the diameter of the stolon along most of its length and not at the swollen areas.

Line M represents the diameter of the stolon at the slightly swollen node region.

Line O represents the height or thickness of the peduncle.

The faintly curving line along the autozooecium suggests the location of the "membranous" area. The scale below belongs with this sketch.

FIGURE 9. Part of a colony showing four autozoids growing quite regularly in pairs, on peduncles at opposite sides of the primary stolon. Two are empty, the third has a setigerous collar and the fourth has a living polypide within. A darker gizzard is evident within the last. The scale directly below belongs to this colony.

FIGURE 10. The section of the stolon showing a septum and the swollen part represented by M in Figure 8. This is the region of the node. Drawn to the scale directly below. Figures 1, 3, 4, 5, and 11 also are drawn to this scale.

FIGURE 11. Part of an autozoid showing the basal region of the setigerous collar through which is lightly indicated the tentacle-bearing lophophore and tentacular sheath. At the base of the setigerous collar are placed two small letters H which represent the width of the tentacular sheath. The measurements are found in Table I. Below the setigerous collar is the vestibular membrane through which are visible muscle fibers. Drawn to the same scale as Figure 10.

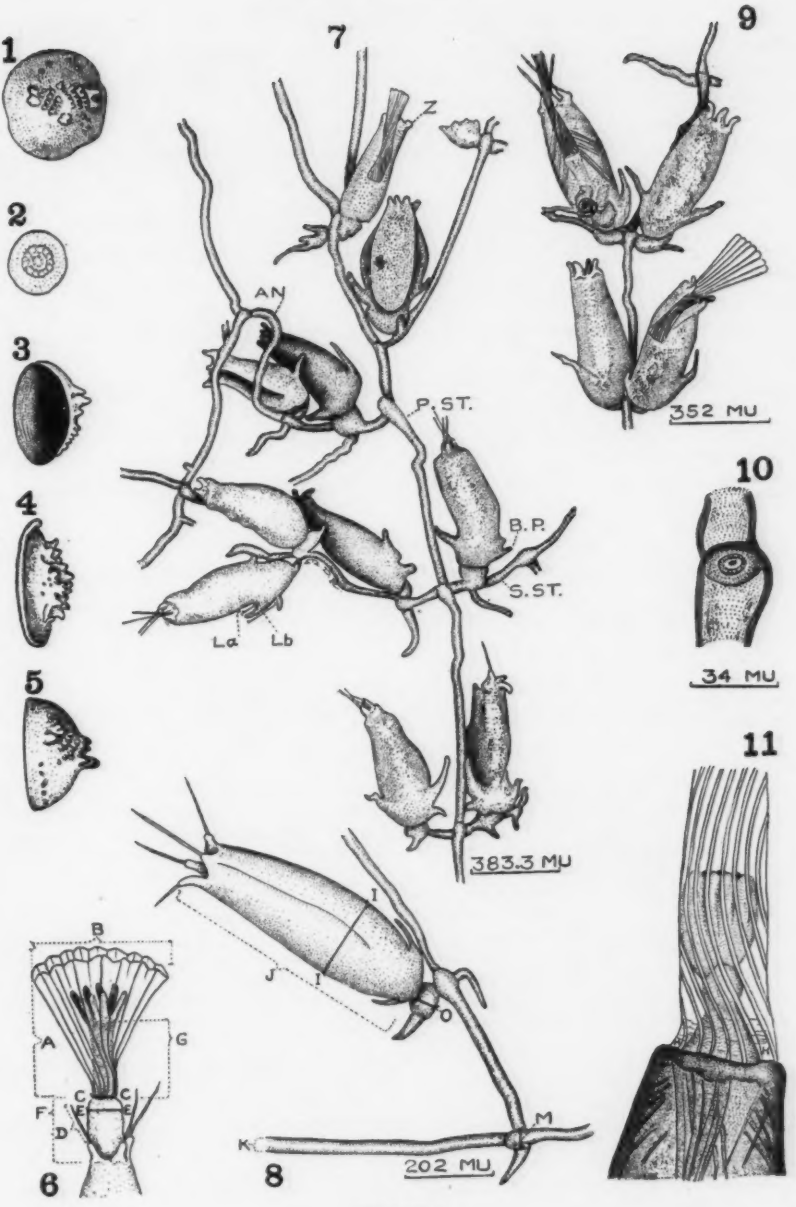
1
2
3
4
5
6
7
8
9
10
11
Z
AN
P. ST.
B.P.
S.ST.
La
Lb
352 MU
34 MU
383.3 MU
202 MU
A
B
C
D
E
F
G
I
J
K
M
O

PLATE I

The second type of structure or possibly individual (?) in *A. setigera* is the peduncle, so designated by Marcus (1937, p. 142). This is a short much swollen segment generally placed between stolons which are at right angles to each other and found at the base of the autozoids (Figs. 8 and 16). It originates from a stolon and gives rise to a stolon and an autozoid. It is cut off from the stolons and autozoid by a uniporous septum. A peduncle is more swollen and of shorter length than the stolon kenozooecium and has a lining membrane. In one instance there appeared a few transverse fibers inside a peduncle.

The third type of individual in an *A. setigera* colony is the autozoid. It arises from the peduncle. The autozoids are just big enough to see with the unaided eye. Harmer (1915, p. 87) gave their length as 0.48–0.55 mm. and Osburn (1940, p. 343) as 0.50–0.60 mm. Their width was given as 0.18 mm. (Harmer, 1915 and Osburn, 1940). Measurements of the Massachusetts specimens are given in Table I.

The autozoids occur in pairs bilaterally placed with respect to the primary stolon (Fig. 9). Where secondary stolons are well developed the autozoids occur in the same manner along the secondary stolon. Occasionally one of the paired autozoids is missing but a stub of its peduncle or a stolon may be present in its place (Fig. 8). These paired autozoids are not truly parallel but converge slightly basally as shown

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PLATE II

All figures are drawn with the aid of a camera lucida and are of *Aeverrillia setigera.*

FIGURE 12. A part of the unfurled setigerous collar, showing the delicate transparent membrane which folds like a fan. Its stiff supporting ribs or setae are transparent also. Drawn to the scale at left.

FIGURE 13. An autozoid in which a very young polypide is growing. A characteristic setigerous collar is not yet present although its Anlage (SC) is visible. Eight tentacles can be counted. The digestive tract is small. A gizzard or proventriculus is present in it. Drawn from living material on August 13, 1944, to the scale shown directly below.

FIGURE 14. Another young autozoid but slightly older than that of the preceding figure. Drawn to the same scale.

FIGURE 15. View of a mature autozoid showing an almost completely retracted polypide, a very long folded setigerous collar partially withdrawn, the U-shaped digestive tract twisted around in the lower half of the zooecium. The gizzard (GZ) is oriented in such a manner that one is looking along its vertical axis. Some of the body wall and polypide musculature is shown, particularly the circularly arranged parietal muscles (PM). The acuminate process is barely visible. Drawn to the same scale as Figure 12.

FIGURE 16. A partly retracted autozoid. The tentacle tips are just barely visible in the dark mass at the base of the spine-bearing processes. The somewhat indistinctly depicted digestive tract is in the basal part of the zooecium. Only a part of the autozoid at left is shown. The scale directly above the setigerous collar applies to this figure.

FIGURE 17. A folded setigerous collar showing typical twisting of the supporting ribs or setae. The transparent membrane is faintly indicated at the distal end. Drawn to the scale directly below.

FIGURE 18. A young autozoid is shown at left. Only a part of the right one is included. The young polypide has eight tentacles and a U-shaped digestive tract. The setigerous collar is not visible but its Anlage (SC) is present. The vestibule (V) and the parieto-vaginal muscles (PVM) are plain. Line N represents the length of the peduncle which bears the autozoid. Measurements of it are given in Table I. Drawn to the same scale as Figure 13, on Aug. 13, 1944, from fresh material.

FIGURE 19. Three of the four chitinized gizzard sconces. The teeth are darker than the rest of the disc in this particular case. Muscle fibers encircle the cluster of four sconces and are here indicated by horizontal or parallel lines. Drawn to the scale at left.

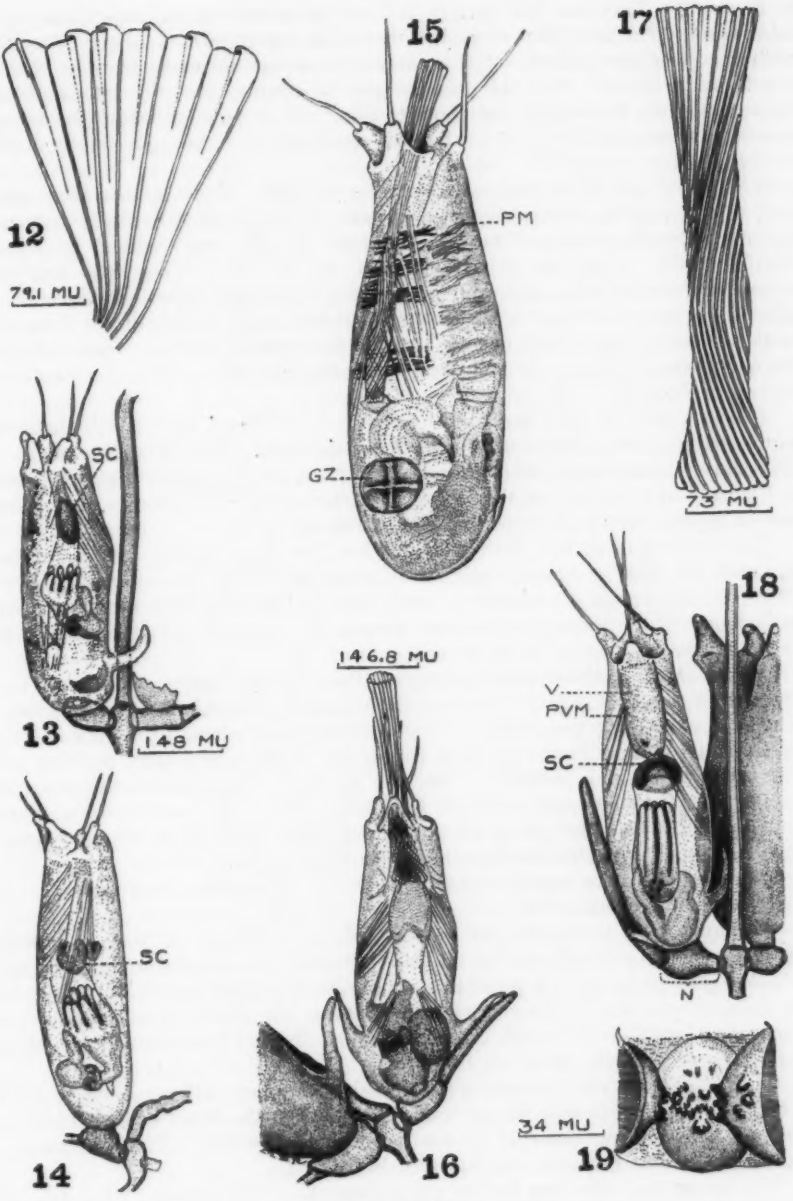
12
79.1 MU
15
PM
GZ
17
73 MU
SC
13
148 MU
146.8 MU
18
V
PVM
SC
N
SC
14
16
34 MU
19

PLATE II

in Figure 9. They are not upright but are recumbent at an angle close to the substratum. The basal part rests directly on the substratum, or is very close to it, while the distal part is free. The autozoids are somewhat elongate ovate with the broad end attached. The side nearest the substratum and the inter-autozoidal stolon is slightly flatter than the opposite side. At its point of origin the autozoid may be globose as in Figure 14 or slightly "stemmed" as in the right-hand individual of Figure 18.

The lower half of the autozoid is swollen slightly. From it arise from one to four, usually two, acuminate clasping processes (Figs. 7 and 16), which were called "tubular adherent processes" by Hincks (1887, p. 128) and "spines" by Harmer (1915, p. 87). They are placed obliquely on the zoid. They may touch the stolons or the neighboring autozoid or else cling to the substratum without touching either the adjacent autozoid or the stolon. Colonies in place on hydroids show some of these clasping processes curling around the hydroid stems, closely adherent. These clasping processes are hollow and not separated by any sort of septum from the rest of the zoid.

Hincks (1887, p. 127) described a large aperture closed by a membranous wall on the greater part of the ventral side of the autozoid. It is difficult to see in the Massachusetts specimens although indications of it are present in Figures 7 and 8. In Figure 7, it is evident on the second and fourth autozoids from the top. Moreover, it appears chitinized rather than membranous.

The distal tapering end of the autozoid has four spine-bearing processes (basal segments or flaps). Occasionally more than four flaps may occur. Harmer (1915, p. 88) reported a specimen with eight. This condition however is very infrequent. These flaps are arranged around the zooecial orifice through which the setigerous collar may be protruded.

The position of these distal triangular flaps is not rigidly, immovably fixed. The line of bending is at the base of the triangle. Sometimes the flaps may be flexed inward so that their spines may cross each other above the orifice as in the top left-hand zoid of Figure 9, or in Figure 16. This is the usual position when the setigerous collar is withdrawn into the autozoid. When the setigerous collar is out the flaps are bent outward as in Figure 6. This is the condition also in many empty zooecia. Whether there are any muscle fibers controlling the movement of these flaps was impossible to determine from the material at hand.

The flaps are more heavily chitinized than the surrounding zooecial wall. The difference is quite noticeable.

The apex of a triangular flap is rounded in all views. A sharply tapering, slightly irregular orificial spine is set shallowly into this rounded area. The spine is hollow, but so far as it is possible to determine its cavity is not continuous with the cavity of the flap but is cut off by a septum. In Porto Rican specimens the spines measured 0.20–0.30 mm. (Osburn, 1940, p. 343). Measurements of Massachusetts specimens are given in Table I.

The setigerous collar is long and very slender when furled. Harmer (1915, p. 88) gives its length as 0.46 mm. and its breadth at the distal end as 0.130 mm. This last figure is undoubtedly of a partly furled individual. The dimensions of the Massachusetts specimens are included in Table I.

The setigerous collar can be protruded clear out of the autozoid (Fig. 6). On the other hand, it also can be completely withdrawn into the autozoid. In fact it

can be pulled in so far that its uppermost or distal tip is halfway down inside the zoid. There are muscular fibers attached to its base (Fig. 11). When it is completely withdrawn the tentacles are below it. When it is protruded and expanded the tentacles are within its circle of setae (Fig. 6).

Hincks (Pl. XII, Fig. 13), Harmer (Pl. V, Fig. 9), Marcus (1937, Pl. XXIX, Fig. 76) and the present writer (Figs. 6, 11, 15, and 17) have pictured the peculiar spiral twisting of the setae of the collar. The setae reinforce a delicate, colorless, transparent membrane which folds neatly like a fan along scarcely discernible creases between adjacent setae, when the collar is being withdrawn (Figs. 6, 12, and 17). The setae or ribs supporting the collar are extremely regular in diameter from base almost to the very tip.

The collar is often found in excellent condition even when all the zoid contents except the zooecial wall have disintegrated.

In young zoids as represented in Figures 13, 14, and 18 the setigerous collar is not yet completed but is represented by a mass of germinative tissue, SC, which temporarily forms a flexible canopy above the tentacles, at the bottom of the vestibule.

The vestibule is the cavity down which the setigerous collar travels when being withdrawn. Its wall is formed by a soft vestibular membrane, to which are attached a number of fibers which constitute the parieto-vaginal muscles. The vestibular membrane is shown withdrawn or introverted in Figure 18 and extruded in Figure 6.

The circular lophophore bears eight tentacles (Figs. 6, 13, and 14). This number is in agreement with the statements of Harmer and Marcus.

The tentacles, when retracted, are pulled into the introverted tentacular sheath in a manner characteristic of the Bryozoa (please compare Figs. 6 and 18).

They surround the entrance to the digestive system which is a U-shaped tract consisting of pharynx, esophagus, proventriculus, stomach and intestine. The most interesting features about the tract are the great length of the esophagus and the presence of a muscular and chitinized proventriculus or gizzard between the stomach and esophagus.

The proventriculi of various species of *Buskia* or *Aeverrillia* are illustrated in papers by Osburn and Veth (1922, Plate I) and Marcus (1941, Plate X, Figs. 44B and 45). Marcus figures the gizzard of both *A. armata* and *A. setigera*. However, the proventriculus of the Massachusetts specimens of *A. setigera* resembles that of his *A. armata* as much as it does that of his *A. setigera*.

The proventriculus of the Massachusetts *A. setigera* is a compact, rounded organ consisting of four conical chitinous sconces capping the internal epithelium. A wide band of circular muscle fibers surrounds these four sconces (Fig. 19). An end view of the proventriculus showing the relation of the four sconces to each other is pictured clearly in Figure 15 and suggested in Figures 9 and 18. A side view, showing the relation of the circular musculature to the sconces and the relative position of the proventriculus in the polypide, is depicted in Figures 13, 14, and 16. A detailed picture of the arrangement of the chitinous and sometimes brown-colored denticles on the sconces appears in Figures 1, 3, 4, 5, and 19. The denticles seem to have a definite arrangement in several roughly V-shaped rows. They are of various sizes. Their color ranges from pale yellow to brown. The shape of each sconce at the base ranges from a broad ellipse to a circle. In side view the sconce

may appear globose, conical, or even slightly flattened, except for the projecting teeth. Careful inspection of an old or empty colony may occasionally reveal sconces of degenerated polypides still within the otherwise empty zooecia. Because the sconces are usually transparent, pale yellow, and small it is easy to overlook them. In degenerating polypides the gizzard can usually be distinguished as the central part of a dark mass of degenerating material.

The relations of the stomach and intestine to the gizzard and to the lophophore can be seen in Figures 13, 14, and 18. In these three instances the digestive tract is empty. In a mature feeding individual the digestive tract is considerably longer, as a study of Figure 15 will show. The intestine opens outside the circle of tentacles—a characteristic of the Ectoprocta.

The musculature of the lower half of the autozoid was difficult to study partly for lack of sufficient living material and partly because in a mature zoid the digestive tract occupies so much of the interior. However Figure 13 does show a suggestion of a band of retractor muscle fibers attached to the base of the tentaculer crown or the upper part of the digestive tract.

The other major muscles attaching to the body wall are the horizontally or circularly arranged parietal muscles. Harmer (1915 p. 88) states that three groups of parietal muscles are visible in his specimens. In the Massachusetts specimens it appears as if there are four groups (Fig. 15).

In a few near-empty zooecia, from which the musculature, tentacles, setigerous collar, and digestive tract were missing but which had a brown body (a mass of dedifferentiating or degenerating tissue) in the upper half of the zooecium, was noticed a rather peculiar globular membranous sac attached to the base of the interior of the autozoid, in the vicinity of the septum which separates the autozoid from the peduncle. This globose mass was hollow. Its wall was soft membranous, and turgid. It is not figured here. Its appearance and position suggest one of two possibilities: 1, it may be a regenerating mass which would give rise to a new polypide within the old zooecium; or 2, it may represent the remains of a degenerating polypide, exclusive of the brown body which was already evident in the upper half of the zooecium. In the fresh water Bryozoa, when polypides of a colony degenerate, sometimes the wall of the colony forms a hollow membranous sac which may either degenerate completely or give rise to a new colony (Rogick, 1938; p. 197).

In studying any form, measurements are extremely helpful. Therefore, as complete a set of measurements of *A. setigera* as was possible was made and is arranged in Table I. The letters and parts A to P are clearly indicated in the drawings of Plates I or II.

## Discussion

*Aeverrillia setigera* seems very widely distributed circumtropically. It has been reported previously from such widely scattered localities as north and east of Australia, China Sea, Gulf of Bengal, Malay Archipelago, Suez Canal, Porto Rico, Brazil's Bay of Santos, etc., whose latitudes range from approximately 24° S to 31° N. The present report extends its range to 41°38′ N. Latitude. A recent report (Hutchins, 1945; from Long Island Sound) cites its occurrence slightly south of the present paper. In spite of this extensive range the number of reports

on the occurrence of this species have not been too numerous: Harmer, Hastings, Hincks, Hutchins, Kirkpatrick, Livingstone, Marcus, Osburn, Thornely, and the present writer.

The Massachusetts specimens agree essentially in measurements and appearance with those found in more southerly waters (Gulf of Bengal, South America, and Porto Rico) by previous workers.

Because of their small size and inconspicuous appearance they are easily overlooked when collecting. Very little is known of their behavior, embryology, life history, and physiology. A study should be made of these as well as of colony degeneration, regeneration, rate of growth, development of the proventriculus and setigerous collar, the location and development of the reproductive system, and the nature of the larva. All the work done so far on this form has been of taxonomic nature. The present paper has added a more complete account of the anatomy, included measurements of a number of parts hitherto unmeasured and added a more complete series of diagrams than have existed previously for this species.

## Summary

1. *Aeverrillia setigera* was found at Woods Hole and at New Bedford, Mass. This extends its northerly range to 41°38′ N. Latitude.

2. The Massachusetts specimens agree closely in appearance and measurements with specimens from more southerly waters of the Gulf of Bengal, Malay Archipelago, South America, and Porto Rico.

3. Measurements of many structures or parts not measured by other workers are here included.

4. The species has been more fully illustrated.

5. The species did not seem to be abundant in the localities from which it has just been reported.

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# STUDIES ON FRESH-WATER BRYOZOA. XVI. FREDERICELLA AUSTRALIENSIS VAR. BROWNI, N. VAR.

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## Introduction

This study deals with a *Fredericella, F. australiensis* Goddard 1909, which was reduced to variety rank and to which were added two other varieties, one of them new. The new variety is here named *F. australiensis* var. *browni,* in honor of Dr. Claudeous J. D. Brown of the Michigan Department of Conservation, Ann Arbor, Michigan, who most generously turned over the material to the author for further study.

The specimens were collected in fair abundance on August 3, 1942, from rocks in an alkali pond about three miles northeast of Church Butte, Uinta County, Wyoming, U.S.A., by Dr. Henry van der Schalie of the University of Michigan, at Ann Arbor.

The writer wishes to express her deep appreciation to both Dr. van der Schalie and Dr. Brown for the opportunity to examine the specimens and to make the present study.

Observations were made on preserved material which was dissected and on preserved material which had to be imbedded and sectioned. No living specimens were available. Dissection and sectioning were necessary to determine tentacle number, diameter of various parts, and internal structure since the zooecial wall was too opaque to permit ready observation of internal structures.

It was necessary to create a new variety, var. *browni*, for the Wyoming form because it resembled very closely in some respects and differed somewhat in other respects from two other forms known heretofore as *Fredericella sultana* subsp. *transcaucasica* Abricossoff 1927 and *Fredericella australiensis* Goddard 1909.

It was necessary to reduce the original *F. australiensis* of Goddard to variety rank, making it *F. australiensis* var. *australiensis* and to add to it two other varieties because the three forms so closely resembled each other and differed noticeably from the long established species of *Fredericella sultana*. Consequently, the former *F. australiensis* Goddard and the *F. sultana* subsp. *transcaucasica* Abricossoff become varieties under the emended *F. australiensis*, namely, *F. australiensis* var. *australiensis* and *F. australiensis* var. *transcaucasica*. The finding of the Wyoming specimens adds a third variety, *browni* to this emended species.

## Fredericella Australiensis, Emended

### *Description*

The colony is attached along the bases of a number of zooecia whose tips become erect at the distal end and eventually give rise to upright branches which usually do not fuse into a solid mass but which form rather openly branched tufts (Fig. 4). Branching is antler-like or very roughly dichotomous. Septa or dissepiments are absent. Zooecial tubes are slightly wider than those of *F. sultana*. The degree of incrustation of the ectocyst varies from almost none in var. *transcaucasica* to a considerable amount in var. *browni* and var. *australiensis*. Floatoblasts are absent. Sessoblasts are rounded or very broadly elliptical, not reniform or very elongate as those of *F. sultana*. They are shorter and broader than those of *F. sultana*. More exact data or measurements will be given in the "Discussion" section. The terms sessoblasts and floatoblasts have been defined in the author's Study XIV. The *F. australiensis* polypides are shorter and stubbier than those of *F. sultana* and are restricted to the zooecial tips whereas those of the latter species are longer and extended further down into the zooecial tubes. The tentacle number is larger in *F. australiensis* than in *F. sultana*. The former has approximately 24 to 30 tentacles while the latter has about 17 to 24 tentacles. The lophophore is decidedly elliptical in var. *australiensis*. In the other two varieties it is uncertain whether the lophophore is nearly circular or definitely elliptical. Living specimens are necessary to determine this point. However, the lophophore is not horseshoe-shaped, except only in the retracted condition. An epistome is present.

*Fredericella australienisis* is characterized by the rounded, broadly elliptical shape of the sessoblasts, the larger number of tentacles and greater zooecial tube diameter, all admittedly somewhat variable characters but unfortunately almost the only ones, barring nature of colony growth and degree of incrustation which in themselves are variable, on which one can make a distinction in this genus.

## *Discussion*

### *Growth habit*

*Fredericella australiensis* and *F. sultana* have a similar growth habit and colonial appearance. The mode of branching is similar. Zoids are adherent for a distance then give off upright branches. Branching is antler-like or very roughly dichotomous in both.

### *Dissepiments or septa*

Allman (1856, p. 112) says of *F. sultana,* "At the origin of the branches there is frequently found a more or less perfect septum." His Plate IX, Figure 3, shows an imperfect or partial septum, i.e., a septum with a hole in it. This chitinous septum is located at the commencement of a branch. Kraepelin (1887) calls the dissepiments rudimentary. In *F. australiensis* there seem to be no septa at the start of the branches. Goddard (1909, p. 490) finds none in var. *australiensis.* Abricossoff (1927b, p. 88) shows none in his Figure 2 of *transcaucasica,* and there appear to be none in var. *browni* (present study).

### *Keel*

There seems to be relatively little difference between *F. sultana* and *F. australiensis* in this character. The zooecial tubes are cylindrical or nearly so in younger *F. sultana* zooecia and keeled in older specimens, so there occur specimens with and without a keel. This is true also of *F. australiensis*—some individuals may have and others may lack a keel.

### *Zooecial tube*

The two species differ very slightly in the shape of the zooecial tubes, when viewed in cross section. The *F. sultana* tubes vary in cross section from cylindrical in unkeeled specimens to somewhat pear-shaped in keeled ones. In *F. australiensis* the tube cross section ranges from an ellipse (in var. *browni,* Figs. 1 and 10) to a rough triangle (var. *australiensis*).

There is a greater difference between the two species in width of zooecial tubes. Those of *F. sultana* are more slender. The diameter of *F. sultana* zooecial tubes of New Rochelle and Lake Erie specimens as given in Study IX (Rogick, 1940, p. 195) ranged from 0.16 to 0.35 mm. and averaged 0.24 mm. for 44 readings. Abricossoff (1927b, p. 91) said that in the U.S.S.R. *Fredericella sultana* the zooecial tube was not more than 0.4 mm. wide. He placed that as the upper limit but did not give the minimum nor average measurements for the point in question. The zooecial tubes of *F. australiensis* are greater in diameter than those of *F. sultana.* Abricossoff (1927b, p. 91) gives the average diameter in *transcaucasica* as 0.5 mm. while the present writer gives a range of 0.259 to 0.576 mm. or an average of 0.391 mm. for the most typical region of a var. *browni* zooecial tube. Thus it would seem that as regards this particular character, var. *browni* is somewhat closer to *F. sultana* than is var. *transcaucasica.*

*Ectocyst*

There is little difference in appearance between the two species so far as chitinized ectocyst is concerned. In *F. sultana* the degree of incrustation of the ectocyst may vary to such an extent that the zooecial tubes may be translucent to opaque, generally favoring the latter. Debris, stone particles and even algae may attach to it. In *F. australiensis* the degree of incrustation varies also from extremely little in var. *transcaucasica* to the usual "opaque," reasonably well incrusted amount in the other two varieties. Sand grains and debris form part of the incrustation. The color of the ectocyst varies from tan to light brown, in *F. australiensis*.

*Polypide*

Kraepelin (1887, p. 99) says that polypides of *F. sultana* are very long. Allman (1856, Pl. IX, Fig. 7) shows such a specimen. In samples observed by various workers, including the present one, the polypides of this species seemed long and slender. On the other hand, in *F. australiensis,* the polypides appear distinctly shorter and stubbier, and are restricted to the zooecial tips (see Goddard, 1909, Fig. 12). Since no digestive tract measurements exist for *F. sultana* it is necessary to judge the relative length of its tract by studying Allman's and other workers' drawings. These measurements would vary with the age and condition of nourishment of the polypides.

*Tentacular crown*

In *F. sultana* the tentacles are long and slender but no measurements exist for them so far as can be determined. In *F. australiensis* the tentacles are generally shorter and stubbier with the possible exception of var. *australiensis*. In the latter variety they measure about one mm. in length and 0.01 mm. in diameter. In var. *browni* the tentacles are shorter and thicker. Unfortunately not too many were in a position to be measured accurately so that one had to depend on the general appearance of those dissected out of the colonies and on a few which were sectioned in the proper plane. These ranged from 0.383 to 0.514 mm. in length and from 0.019 to 0.029 mm. in width (Table II). This is shorter and wider than in var. *australiensis*. No measurements are available for var. *transcaucasica* tentacles. One has to judge them from Abricossoff's (1927b, p. 88, Fig. 2) figure in which they appear shorter and stubbier than tentacles of his *F. sultana* (*ibid.*, Fig. 1).

The number of tentacles does not seem to vary as much in *Fredericella* individuals as it does in those of *Plumatella* and *Hyalinella*. In *Hyalinella punctata,* the author (1945, Study XV. p. 69) found that the ancestrula or first polypide of a colony could be distinguished from successive polypides on the basis of the number of tentacles. It had about 10± less than successive polypides did. Whether the same general principle holds for *Fredericella* and other fresh-water forms could easily enough be determined by germinating statoblasts of the various forms and keeping accurate counts of the number of tentacles developed in each zoid.

The tentacle number of the two species of *Fredericella* is different. In *F. sultana* it ranges from 17 to 24, with 20 to 22 being the most common number. In *F. australiensis* the number ranges from 24 to 30.

TABLE I

*Comparison of the three varieties of Fredericella australiensis and including Borg's African specimens*

| Part or structure | var. *australiensis* | var. *browni* | var. *transcaucasica* | Borg's African specimens |
|---|---|---|---|---|
| A. Lophophore x-section<br>1. expanded?<br>2. retracted | about 0.38×0.23 mm. | 0.182×0.133 mm. average | no data given | no data given |
| B. Sessoblast length and width | no data given | 0.382 × 0.316 mm. average | 0.470×0.315 mm. average | Type A sessoblasts, 0.37–0.43 mm. long by 0.22–0.27 mm. wide<br>Type B sessoblasts, 0.33–0.40 mm. long by 0.30–0.35 mm. wide |
| C. Zooecial tube diameter | no data given | 0.391 mm. average | 0.5 mm. average | Creeping part of tube, 0.33–0.45 mm.<br>Erect part of tube, 0.24–0.33 mm. |
| D. Tentacle number | 28–30 | 24–28 | no data given | 24–28; usually 26–27 |
| E. Ectocyst appearance | chitinous, brown, incrusted | chitinous, well incrusted, tan, quite opaque | chitinous, thick, light brown, transparent, very little incrusted | chitinous, considerably incrusted with sand grains |
| F. Zooecial tube in x-section | roughly triangular | elliptical | no data given | Some strongly keeled. Triangular in attached parts of colony and rounded in erect part of colony |
| G. Polypides | "seen only at ends of filaments"—Goddard, so probably were short | short and stubby; at tips of zooecial tubes | short and stubby | sometimes arc-shaped when retracted |
| H. Source of above information | Goddard, 1909 | Rogick, present study | Abricossoff, 1927b | Borg, 1937 |

Previous authors have given ample data on the tentacle number of *F. sultana*. Allman (1856, p. 112) states that this species has about 24 tentacles. His Plate IX, Figure 2, shows 20 to 24 tentacles on various polypides while his Figure 7 (same Plate) shows 25. Nowhere does he call attention to this large number however. Hyatt's (1868, p. 220) *F. regina*, now a synonym for *F. sultana*, had 18 to 22. Kraepelin's (1887, pp. 92, 103) specimens had 20 to 22 as a rule but could also range from 18 to 24. Braem's (1890, p. 11) ranged from 20 to 22, with one specimen being found which had only 17. Toriumi's (1941, pp. 196-197) had 17 to 23. The present writer has found New Rochelle specimens with 24 (1940, Study IX, p. 195), Lake Erie specimens showing the full range of 18 to 24, but usually with 20 to 22 tentacles (1935, Study II, p. 250).

Borg (1937, pp. 272-275) reported the collection of a *F. sultana*, from the Sahara region of Africa, which had 24 to 28 tentacles, wider zooecial tubes than the

## EXPLANATION OF PLATE I

All figures are of *Fredericella australiensis* var. *browni* from the Wyoming collection and have been drawn with the aid of a camera lucida.

FIGURE 1. Cross section through a sand and debris incrusted zooecial tube near the tip, which at this level contains the retracted tentacular crowns of two polypides. The tentacular crown at left has been sectioned through the lophophore region at the bases (TB) of the tentacles and through the epistome (EP). The lophophore bears 25 tentacles in this specimen and their bases (TB) at this level are somewhat triangular. The heavy staining of the nuclei accounts for the darkest wavy "stratum" of the tentacles. The lightly stippled material immediately on either side of this dark nuclear "line" or "band" is cytoplasmic material. At this level the surface of the tentacles facing the epistome is ciliated but that is not shown on the drawing. The tentacular crown at left appears to be horseshoe-shaped but that is because it is in the retracted condition. Such a condition also occurs in a retracted *F. sultana* polypide (see Braem, 1890, Pl. V, Fig. 68). The group of 27 tentacles (T) at right belongs to a second polypide. The tentacular cell nuclei are more conspicuous on the inner border of each tentacle where the cells are taller and closer together than on the outer border where the cells are flatter. By inner border is meant the surface facing the epistome and by outer border is meant the surface at the periphery of the tentacular crown. That orientation is best noted in the outermost circle of tentacles. Those within the circle are less regularly oriented. Here again, the cilia have been omitted from the drawing. The zooecial tube is a somewhat longer ellipse here near the zooecial tip than at a level lower down along the tube, as shown in Figure 10. The wall of the tube varies in thickness because of the incrustation. Drawn to Scale A which is 0.073 mm. long.

FIGURE 2. A branch from a colony, showing the zooecial tubes (Z) closely adherent to the substratum (SM), which in this instance is blacked in. The tips of the zooecia are not generally attached to the substratum but are free and directed upward. The condition of the tips indicates that all the polypides are retracted. Drawn to Scale C.

FIGURE 3. Six sessoblasts (SS) shown inside the thin, translucent, tubular, cellular or membranous endocyst (EN). The ectocyst has been removed from the specimen. The cement ring is the darkest part of the sessoblast here. Three of the sessoblasts are turned a little so that one edge shows, but the other does not. The endocyst was torn at the right during dissection and the right-hand statoblast is partly out of it. Drawn to Scale B, which is 0.3 mm. long.

FIGURE 4. Habit sketch of a part of a colony or zoarium showing the adherent base, the upright branches and the mode of dichotomous branching. The substratum is shown in black. When the zooecial tips appear as in this figure their tentacles are either generally retracted or else the tips may be empty. It is sometimes hard to tell if the colony has polypides within it or not because the ectocyst is fairly opaque, so that only very dark structures like the sessoblasts are perceptible with any ease. Since polypide parts are light in color they usually do not show through the ectocyst but have to be dissected out for study. If a colony has been empty a long time the zooecial tips may be broken off and then their emptiness, of course, is evident. Drawn to Scale C which is equivalent to one mm.

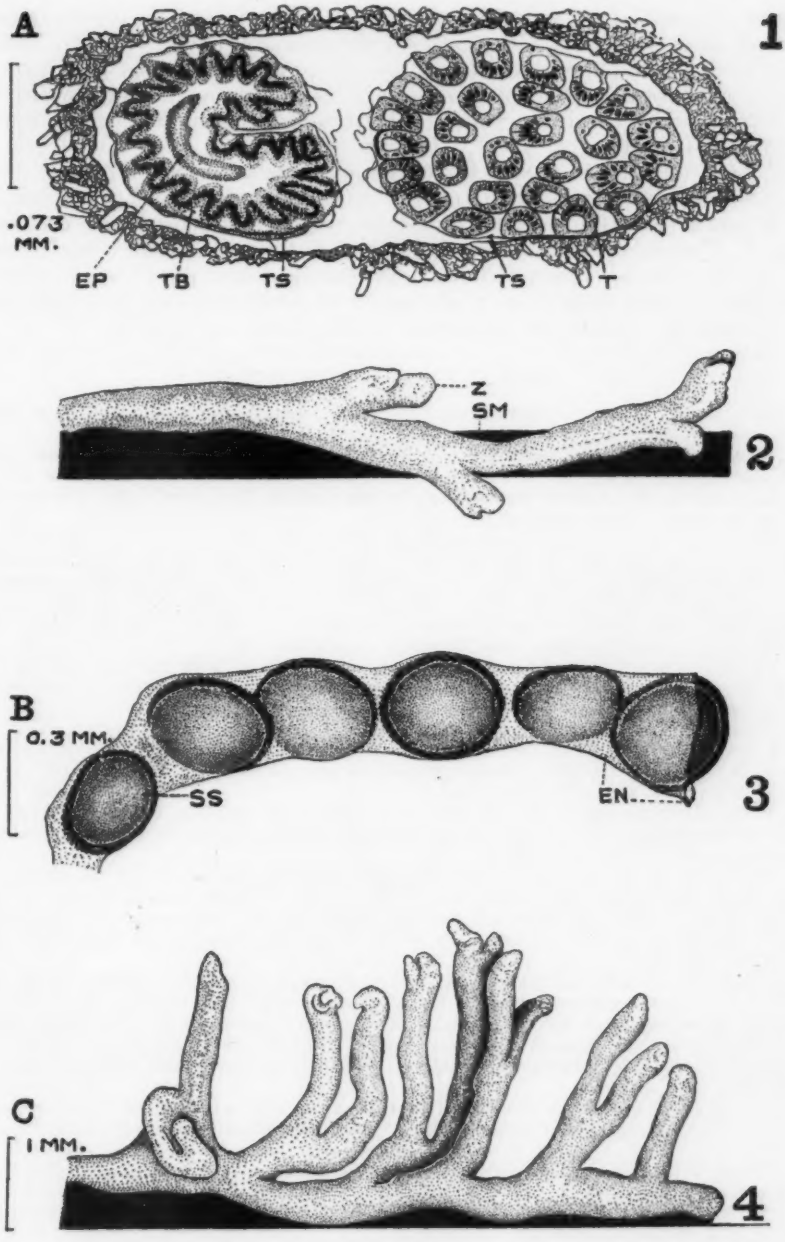


PLATE I

ordinary *F. sultana,* and statoblasts which were extremely variable (Table I) and in many cases rounded or oval. Some of his specimens (Borg, 1937, Pl. XVII, Figs. 2 and 3) look very much like *F. sultana* and probably are but his Figure 1 (same plate) appears definitely to belong to *F. australiensis.* Judging by tentacle number, zooecial tube diameter, and appearance of the pictured statoblast inside its tubes, it seems to agree favorably with var. *browni.*

Borg (1937, p. 275) also mentions very incidentally another interesting form of *Fredericella, F. sultana* forma *major,* from the north of Sweden, which has 28 to 32 tentacles and is generally of a greater width (presumably zooecial tube width). This would be in conformity with *F. australiensis.* Unfortunately however, he gives no description, pictures, or dimensions of it so that its status is quite uncertain. It may either prove a new species of *Fredericella* or a new variety of *F. australiensis.* At any rate it would be worth a fuller investigation.

## EXPLANATION OF PLATE II

These are all figures of *F. australiensis* var. *browni* (from the Wyoming locality) and were drawn with the aid of a camera lucida.

FIGURE 5. Surface view of the greater part of one fairly young completed sessoblast. The chitinous substance of the valve gradually thins out toward the center which part is the last to be closed over by the chitin in development. In this specimen the central region was thinnest and palest in color. Drawn to Scale H.

FIGURE 6. An abnormally shaped sessoblast. There were relatively few mis-shapen sessoblasts found in the collection and this was one of them. Its drawing is included as a contrast to the typical sessoblasts shown in Figures 9 and 11. The sessoblast valves are joined together at the border in what is sometimes called a cement ring (CR). The sessoblast contains opaque germinative material (GM) occupying almost all the space between the two capsule valves. The cement ring is dark amber color while the valves are a paler amber.

FIGURE 7. A tentacular crown dissected from a zooecial tube, from preserved material. It shows the relative length of the tentacles. The tentacular mass was slightly disarranged during dissection. Drawn to Scale D whose length is given below the figure.

FIGURE 8. A side or edge view of a sessoblast. The two irregular dark patches (CH) on one valve are chitinous material which grows on some of the sessoblasts, attaching them to the substratum, or to the wall of the colony. A face view of a similar growth is shown in Figure 9. Drawn to the same scale as Figures 9 and 11.

FIGURE 9. A portion of the cellular endocyst tube (EN) enclosing a sessoblast (SS) on which are growing several irregular or crescent-shaped patches of chitin (CH). The sessoblast is typical, normal. Drawn to Scale E.

FIGURE 10. A cross section of a zooecial tube taken about midway between the tip and the base, shown in silhouette. This section is more typical of the elliptical shape of the ectocyst tube than is Figure 1, which was taken near the tip which housed the broadest part of the polypides. The irregularity of the zooecial wall is due to the material incrusting it (see Figs. 1 and 14). Drawn to Scale F.

FIGURE 11. A sessoblast showing the internal germinal mass (GM) shining through the deep amber-colored translucent capsule. The colors of the rest of the sessoblast at the line of junction of the two valves are as follows. The outermost stippled ring is dark reddish amber while the ring shown in black is a very dark brown. These two dark outer bands represent the cement ring area. The shape of the sessoblast is typical for this variety and species. Drawn to Scale E.

FIGURE 12. Surface view of a portion of a sessoblast valve which is older than that portrayed in Figure 5. A delicate raised chitinous tracery, here shown in black, covers it. Drawn to Scale G.

FIGURE 13. Surface view of a portion of still older sessoblast valve than shown in Figure 12. The raised tracery is coarser, darker, and more prominent. Drawn to Scale G.

FIGURE 14. Surface view of ectocyst showing the minute sand grains and other debris imbedded in it. Drawn to Scale H.

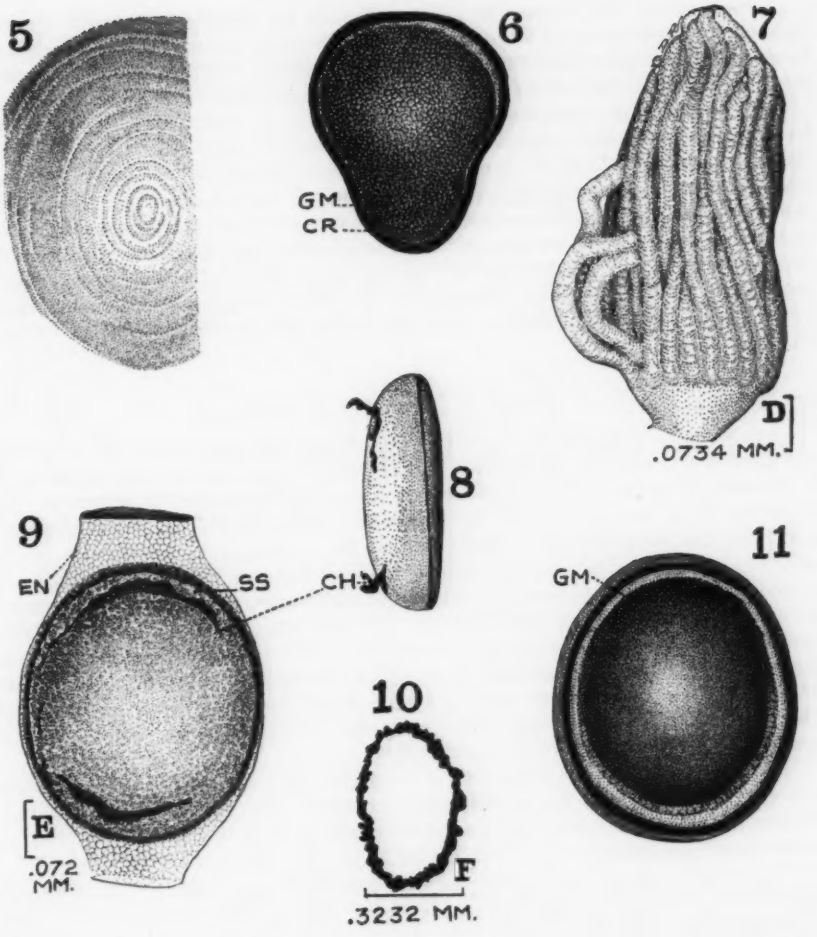
5
6
GM.
CR.
7
D
.0734 MM.
8
9
EN
SS
CH.
E
.072 MM.
10
F
.3232 MM.
11
GM.
12
G
.051 MM.
13
.0.1 MM.
14
H

PLATE II

Borg mentions that Kraepelin (1914, reference not available to present author) has collected specimens of *Fredericella* from Rhodesia, Africa, which have statoblasts which are about one third smaller than ordinary German *F. sultana* specimens. Nothing is said about the number of tentacles in the Rhodesian form.

The shape of the expanded tentacular crown in *F. sultana* is nearly circular. In *F. australiensis* var. *australiensis* the lophophore is very definitely elliptical in shape, measuring 0.23×0.38 mm. In var. *browni,* it can not be said for certain what the shape is in expanded lophophores since all were retracted in the material studied. Abricossoff makes no mention of this point in var. *transcaucasica.* Cross sections of retracted *F. sultana* and *F. australiensis* look similar except that the latter species has a greater number of tentacles. When the polypides of both species are withdrawn, their lophophores assume a crescent or horseshoe shape (Fig. 1 of present study; Braem, 1890, Pl. V, Fig. 68; Goddard, 1909, p. 491 and Pl. XLVII, Fig. 5).

*Sessoblasts*

Statoblasts are extremely important in identification of fresh-water Bryozoa, but those of *Fredericella, Plumatella,* and *Hyalinella* often are not entirely adequate in themselves, especially when present in very small numbers, to determine the exact variety or sometimes even the species to which they belong. It is necessary that sufficient specimens be available so that the normal type of statoblast can be observed, for there is so much variation in shape and size that one can readily be misled by examination of just one or two lone statoblasts. There is a great amount of intergradation between statoblasts of different varieties and species. Almost every worker has rather helplessly commented on the fact, yet has been unable to find a criterion that is invariable by which to identify the species and varieties. Statoblasts alone of the above forms are often insufficient for absolute identification. One should also have the colonies and polypides, living and preserved, in sufficient quantity to really make accurate identifications.

In *Fredericella* there is apparently a complete series of intergrading sessoblasts between *F. sultana* and *F. australiensis.* However, the vast majority of the *F. sultana* statoblasts are reniform or quite elongated while the majority of the *F. australiensis* sessoblasts are more rounded or broadly elliptical in outline.

The extreme dimensions for *F. sultana* sessoblasts are: length range from 0.27 to 0.57 mm. and width range from 0.139 to 0.37 mm. The minimal figures above are from some Lake Erie specimens (Rogick, 1935, Study II, p. 250) and the maximal figures are for some European specimens (Kraepelin, 1887, p. 104). As a rule, the average length and width figures show that *F. sultana* sessoblasts are considerably longer than wide, a fact that can not always be fully appreciated from lone maximum and minimum figures. The extreme dimensions, so far determined for *F. australiensis* sessoblasts, are: length range from 0.331 to 0.470 mm., width range from 0.267 to 0.367 mm. if var. *browni* and var. *transcaucasica* (Tables I and II) are considered, or 0.22? to 0.367 if Dr. Borg's African specimens are included in these computations and if the African forms should all prove to belong to F. *australiensis* and not to *F. sultana.* The reason for the question mark after 0.22 in the preceding sentence is that this particular measurement may or may not have been of this species or variety. The average length and width of *F. australiensis* statoblasts,

TABLE II

*Measurements of Fredericella australiensis var. browni from Wyoming*

| Part or structure | Maximum | Minimum | Average | Number of readings |
|---|---|---|---|---|
| A. Sessoblast | | | | |
| 1. Total length | 0.461 mm. | 0.331 mm. | 0.382 mm. | 69 |
| 2. Total width | 0.367 mm. | 0.266 mm. | 0.316 mm. | 69 |
| 3. Thickness in middle | | | 0.101 mm. | 1 |
| 4. Cement ring diameter | | | 0.014 mm. | 1 |
| B. Zooecial tube diameter along the longer of the two transverse axes | 0.576 mm. | 0.259 mm. | 0.391 mm. | 50 |
| C. Tentacles | | | | |
| 1. Number | 28 | 24 | 26–27 | 26 |
| 2. Length | 0.514 mm. | 0.383 mm. | 0.451 mm. | 3 |
| 3. Broadest part of the shorter transverse diameter | 0.029 mm. | 0.020 mm. | 0.025 mm. | 10 |
| 4. Longer transverse diameter (at right angles to preceding measurement) | 0.027 mm. | 0.019 mm. | 0.024 mm. | 14 |
| D. Lophophore retracted within zooecial tube: | | | | |
| 1. Antero-posterior diameter | 0.308 mm. | 0.147 mm. | 0.182 mm. | 8 |
| 2. Lateral diameter | 0.170 mm. | 0.111 mm. | 0.133 mm. | 8 |
| E. Epistome | | | | |
| 1. Antero-posterior diameter | | | 0.019 mm. | 1 |
| 2. Lateral diameter | | | 0.056 mm. | 1 |
| F. Esophagus | | | | |
| 1. Length | | | 0.193 mm. | 1 |
| 2. Width | 0.060 mm. | 0.051 mm. | 0.054 mm. | 3 |
| G. Stomach | | | | |
| 1. Length | 0.653 mm. | 0.634 mm. | 0.644 mm. | 2 |
| 2. Width | 0.070 mm. | 0.066 mm. | 0.068 mm. | 2 |

at least of the *browni* variety, show that the statoblasts are more nearly a broad ellipse than are those of *F. sultana*. The *F. australiensis* sessoblasts are generally slightly flattened on one side and very probably roughened by various markings on the other, when mature (Figs. 12 and 13). Neither Goddard nor Abricossoff mention the nature or pattern of the surface markings on their specimens' sessoblasts. Variety *browni* however had some sessoblasts with markings (Figs. 12 and 13); so does *F. sultana* (Rogick, 1937, p. 102, Fig. 1).

### *Distribution*

*Fredericella australiensis* has a widely scattered distribution although it has been reported relatively few times. Its three varieties are distributed as follows. Variety *australiensis* occurs in the water supply system at Pott's Hill in New South Wales, Australia (Goddard 1909, pp. 487-489). Goddard reported that the *F.*

*sultana* recorded earlier from Australia by Whitelegge is probably his own *F. australiensis*. Variety *transcaucasica* occurs in Lake Madatapeen, Tiflis District, the Transcaucasus, in the U.S.S.R. (Abricossoff 1927a, p. 308 and 1927b, p. 91). This variety was collected by B. S. Winograd on July 1, 1915 and later identified by Dr. Abricossoff. Variety *browni* occurs in Uinta County, Wyoming, U.S.A. Some of Dr. Borg's material from rivers in the Sahara region of North Africa is very likely *F. australiensis* var. *browni*. This widens the distribution of *F. australiensis* to 4 ? continents: Africa?, Australia, Eurasia, and North America.

### *Key to Varieties of Fredericella australiensis*

1 (2) Chitinous ectocyst well incrusted with sand grains and debris; rather opaque.....3
2 (1) Chitinous ectocyst very little incrusted; very transparent; zooecia about 0.5 mm. wide; sessoblasts average 0.315 × 0.47 mm.....var. *transcaucasica*
3 (4) Tentacle number 24–28; sessoblast average 0.316 × 0.382 mm.; zooecial tubes elliptical in cross section.....var. *browni*
4 (3) Tentacle number 28–30; zooecial tubes roughly triangular in cross section var. *australiensis*

## Fredericella Australiensis var. Browni, New Variety

### *Description and Discussion*

This variety is illustrated in Figures 1 through 14. Its measurements are given in Table II. Its points of difference and resemblance as compared with the other two varieties are briefly summed up in Table I. Some gaps exist in the information about this variety and they are: 1, the shape and dimensions of the expanded lophophore and 2, the unavailability of living specimens for a more complete study of tentacle and polypide size and variation. However, on the basis of the preserved material available, the following description of the variety can be made.

Variety *browni* has a thin chitinous ectocyst well incrusted with sand grains and debris (Figs. 1 and 14). It is of light tan color and rather opaque. The opacity of the zooecia is such that it is possible to see whether the much darker colored sessoblasts are present, but not whether polypides are present because the light color of the polypides blends in so well with the color of the incrusted ectocyst. To determine if tubes contain polypides it is frequently necessary to tear them apart. Only then are the polypides visible.

Basal zooecia are recumbent or adherent in their more proximal part, with the tips directed upwards (Figs. 2 and 4). From these arise erect branches (Fig. 4). The zooecia are generally elliptical in outline (Figs. 1 and 10). Occasionally a faint keel may be present (Fig. 2) but usually it is not noticeable. The colony appears upon rocks as a coarse tracery or tufted mass, depending upon the number of polypides in it. If the number of polypides is small or if the periphery of the colony is examined there will be located the more adherent members. If the colony is luxuriantly branched and on a rather limited substratum then it has many more upright branches. These are not fused together but retain their individuality and open mode of branching. The zooecia are usually very long (Fig. 4). The ectocyst has considerable rigidity and firmness. The zooecia are somewhat wider than in *F. sultana*. Those of var. *browni* are not as wide apparently as those of var. *transcaucasica* (Table I). The ectocyst is too opaque to be able to see dissepiments or incomplete septa at the commencement of the zoids even if they

were present in this variety. Such dissepiments occur in *F. sultana*. A diligent search was made through sectioned and dissected *F. australiensis* var. *browni* material but no dissepiments could be found.

The ectocyst is lined with a soft thin transparent membranous endocyst. The endocyst encloses the polypides and sessoblasts (Figs. 3 and 9).

The polypides of var. *browni* appear short and stubby. The tentacles, especially, seem so, perhaps because of their considerable number, 24–28 (Fig. 7). The tentacles ranged in number from 24 to 28 but the usual number was 26 or 27, just as Borg had found in his African specimens. Of course, the condition of the colony, the length of the polypides and tentacles are greatly influenced by the state of nutrition of the colony. The better fed the colony, the longer the polypides and tentacles. However, the var. *browni* specimens seemed well enough nourished. Their digestive tracts were well filled with algal food.

The parts of the digestive tract are the same as for *F. sultana* and *Plumatella repens*—ciliated mouth guarded by the epistome, ciliated pharynx, esophagus, stomach, and intestine.

The reproductive organs were not observed.

The sessoblasts of var. *browni* are generally smooth on one side (Fig. 5) and roughened on the other (Figs. 12 and 13). However, some older sessoblasts may show roughening or markings on both sides, and in addition, chitinous material may begin to grow on the valve of the statoblast (Fig. 8), attaching it to the endocyst (Fig. 9) or to the body wall and possibly eventually to the substratum.

Variety *browni's* sessoblast shape is best shown in Figures 9 and 11, which are typical. Abnormal specimens occasionally occur and one such is shown for contrast in Figure 6.

The colors of the sessoblasts range from reddish yellow to brown, depending upon the age; the older, the darker.

There were quite a number of sessoblasts present in the zooecial tubes of the Wyoming specimens at the time of collection (August).

The sessoblasts were so distinctive in shape and general proportions that it was immediately evident that one was not dealing with *F. sultana* but with a form related to Abricossoff's and Goddard's specimens—a distinct species—*F. australiensis*.

The decision to make each of these forms (*F. australiensis, F. sultana transcaucasica,* and the Wyoming specimens) a separate variety of *F. australiensis* was based on the great similarity to each other so far as the shape of their statoblasts was concerned and their slight but distinct differences as regards the nature of the ectocyst and the number of tentacles (refer to Key to Varieties and Tables I and II).

## Summary

1. The species *Fredericella australiensis* has been emended to include three varieties.

2. A new variety, *F. australiensis* var. *browni,* has been erected.

3. Two other previously recorded forms, *F. australiensis* Goddard 1909 and *F. sultana* subsp. *transcaucasica* Abricossoff 1927 have been reduced to the status of varieties under the emended *F. australiensis.*

4. The emended *F. australiensis* is characterized by its rounded or broadly

elliptical sessoblasts, its wider zooecial tubes, its greater tentacle number, its lack of dissepiments and the shorter stubbier tentacles and polypides which are generally confined to the tips of the tubes. These features distinguish it from *F. sultana*.

5. The varieties *australiensis, browni,* and *transcaucasica* are placed in *F. australiensis* because they possess the above characteristics.

6. The three varieties are distinguished from each other on the basis of degree of incrustation of their ectocyst, the difference in number of tentacles, appearance of the zooecial tubes in cross section and miscellaneous measurements.

7. *Fredericella australiensis* has a wide but scattered distribution. It is represented in Australia by var. *australiensis*; in Eurasia (the U.S.S.R.), by var *transcaucasica,* in Africa?; and in North America, by var. *browni.*

8. The specimens which were immediately responsible for the erection of the new variety, *F. australiensis* var. *browni,* were obtained through the kindness of Dr. C. J. D. Brown and Dr. H. van der Schalie of Ann Arbor, Michigan, who turned the collection over to the author for study. The specimens were collected by Dr. van der Schalie on August 3, 1942, from rocks in an alkali pond about three miles northeast of Church Butte, Uinta County, Wyoming, U.S.A.

9. The study includes 14 illustrations and one table of measurements dealing with var. *browni* and one table of comparison between the three varieties.

10. A brief summary of available measurements and other data on *F. sultana* is given.

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# STUDIES ON THE BIOCHEMISTRY OF TETRAHYMENA. VII. RIBOFLAVIN, PANTOTHEN, BIOTIN, NIACIN AND PYRIDOXINE IN THE GROWTH OF T. GELEII W

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With the substitution of chemically known materials for all but one fraction in the medium for the growth of Tetrahymena it has been possible to determine with some degree of exactness the specific vitamin requirements of this important ciliate. When proteins, such as casein or gelatin, or peptones are used as the base medium it has been impossible to determine the importance of those vitamins which were stable to treatments which would not also destroy other essential materials. Using these types of media, claims have been made for the essential nature of thiamine and of riboflavin for *Tetrahymena geleii* (Hall and Cosgrove, 1944; Hall, 1944). It was earlier indicated (Kidder and Dewey, 1942) and later conclusively proven (Kidder and Dewey, 1944; 1945a; 1945b) that at least eight strains of Tetrahymena could grow in a medium in which the thiamine had been destroyed.

When it was found that *T. geleii* could be grown successfully in a mixture of amino acids (Kidder and Dewey, 1945c) and that two of the three "unknown growth factors" could be replaced with nucleic acid derivatives (Kidder and Dewey, 1945d) and that the remaining "unknown growth factor" (Factor II) was relatively stable and was not adsorbed readily on activated charcoal, it became possible to examine the effects of the omission of a number of the vitamins. Hitherto these vitamins had been added routinely to guard against the possibility of any one of them proving to be a limiting factor. It was found (Kidder, 1945) that folic acid is an essential growth factor for *T. geleii* W, this fact being obscured previously by the necessary inclusion of Factor I (containing folic acid) as the lead acetate precipitate fractions of raw materials, the Factor I activity being readily absorbable on activated charcoal.

The present work has been made possible by the utilization of a number of different treatments of the Factor II preparations and the inclusion of all other constituents of the medium as chemically pure materials. Furthermore, this work would not have been possible without the employment of a microbiological method for the detection of traces of the growth factors under consideration. We have utilized *Lactobacillus casei* as a tool in this study, and while we have made no attempts to assay various preparations quantitatively, we have used the bacterial method for determining the total lack of the vitamin under immediate consideration. It has been possible also, to show that the ciliate possesses the ability to synthesize certain of the B vitamins, by determining the increase of the vitamin by the *L. casei* test after the growth of the ciliate.

[1] Aided by grants from the Morgan Edwards Fellowship Fund and the Manufacturers Research Fund for Bacteriology and Protozoology of Brown University. Present address Biological Laboratory, Stanford University.

## Materials and Methods

### *Organisms*

The ciliate used in this study was *Tetrahymena geleii* W, which has been maintained in pure (bacteria-free) culture in this laboratory for a number of years and which has been used in numerous previous studies (Kidder and Dewey, 1942–1945). The organism has been grown in amino acid media for the past one and one-half years and all inocula for the present series were taken from these stocks.

*Lactobacillus casei* 912 was used for the microbiological testing of experimental media. This organism was obtained from the Squibb Institute for Medical Research through the courtesy of Dr. Vincent Groupé. Stocks were carried in yeast extract-dextrose-agar stab cultures, transplants being made at monthly intervals, incubated at 37° C. for 24 hours and then placed in the refrigerator.

### *Ciliate base medium*

One type of base medium was used routinely. This appears in Table I with the complete supplements. Each vitamin under investigation was omitted from the medium separately.

### *Preparation of Factor II*

It has been necessary to treat the Factor II preparations in various appropriate ways in order to eliminate the different vitamins studied. In the earlier work (Kidder and Dewey, 1945d) the prime consideration in the Factor II preparation was the elimination of Factors I and III activity, and the methods used did not necessarily render the preparation vitamin free. In this study the inclusion of Factor I and Factor III activity was of no particular importance, and so attempts were made to eliminate the vitamin under consideration and still retain maximum Factor II activity. This latter was not always possible as some of the treatments used not only removed or destroyed the vitamin but also lowered the Factor II activity. Nevertheless preparations which were satisfactory for this study were obtained, and these will be described under the heading of each vitamin.

### *Riboflavin-free preparation (8L531).*

Liver Fraction L [2] (50 grams) was dissolved in one liter of distilled water and a 40 per cent solution of normal lead acetate was added until no more precipitate formed. The precipitate was removed by filtration with the aid of Celite and discarded. The filtrate was neutralized with NaOH and treated with an excess of basic lead acetate. The second precipitate was removed and discarded, the excess lead removed with 9 per cent oxalic acid and the excess oxalic removed as the oxalate with $Ca(OH)_2$. Tests at this stage showed the presence of large amounts of riboflavin, but after adsorption with 10 grams of Norit A for one hour at room temperature at pH 3.5 the riboflavin had been quantitatively removed. This preparation was used in a concentration of one part in ten parts of final medium.

[2] Furnished through the courtesy of Dr. David Klein and the Wilson Laboratories.

TABLE I

*Base Medium*

| | mg./ml | | micrograms/ml. |
|---|---|---|---|
| *l*(+)-arginine mono-hydrochloride | 0.82 | biotin methyl ester[3] | 0.00005 |
| *l*(−)-histidine mono-hydrochloride | 0.10 | calcium pantothenate[3] | 0.10 |
| *dl*-isoleucine | 0.35 | thiamine hydrochloride | 0.10 |
| *dl*-leucine | 0.35 | nicotinamide[3] | 0.10 |
| *dl*-lysine | 0.60 | riboflavin[3] | 0.10 |
| *dl*-methionine | 0.34 | pyridoxine hydrochloride[3] | 0.10 |
| *dl*-phenylalanine | 0.14 | *p*-aminobenzoic acid | 0.10 |
| *dl*-serine | 0.04 | *i*-inositol | 1.00 |
| *dl*-threonine | 0.20 | choline chloride | 1.00 |
| *l*(−)-tryptophane | 0.10 | folic acid concentrate[4] | 1.00 |
| *dl*-valine | 0.20 | | mg./ml. |
| dextrose | 2.00 | hydrolyzed yeast nucleic acid[5] | 0.05 |
| $MgSO_4.7H_2O$ | 0.10 | | |
| $K_2HPO_4$ | 0.10 | | |
| $CaCl_2.2\ H_2O$ | 0.05 | Factor II preparation (see text) | |
| $FeCl_3.6\ H_2O$ | 0.00125 | | |
| $MnCl_2.4\ H_2O$ | 0.00005 | | |
| $ZnCl_2$ | 0.00005 | | |

*Pantothen-free preparation (8L531H).*

Although pantothenic acid is adsorbed on activated charcoal the time and temperature allowed in preparing the riboflavin-free medium is insufficient for the complete removal of pantothen. Raising the temperature, increasing the time, or increasing the amount of Norit used was not practical as the Factor II activity was greatly reduced (Kidder and Dewey, 1945d). Therefore advantage was taken of the sensitivity of pantothenic acid to alkali and heat and the riboflavin-free preparation was adjusted to pH 10.0 with NaOH and autoclaved for two hours. The Factor II activity was somewhat reduced by this treatment, but the preparation was entirely satisfactory for use. *L. casei* tests showed that the pantothenic acid content had been lowered to an insignificant amount. This preparation was used in concentrations of one part in ten parts of final medium.

*Biotin-free preparation (8L5C1).*

The most active biotin-free preparation, and therefore the one used in this study, was made in the following manner. Ten grams of Liver Fraction L was dissolved in 200 ml. of distilled water and brought to boiling. To this boiling mixture were added 10 ml. of a 10 per cent solution of $NaHSO_3$ and 10 ml. of a 10 per cent solution of $CuSO_4$, and boiling was continued for 3–5 minutes. The precipitate was removed on a fluted filter and the process repeated once. The copper was removed as CuS after treating with 15 per cent $Na_2S$ and the sulfate and sulfite removed as the barium salts after treatment with $Ba(OH)_2$. The volume of the filtrate was reduced to 200 ml. and a 100 ml. aliquot was adjusted to pH 3.5. Two grams

[3] Omitted singly in the appropriate series of experiments.

[4] The folic acid concentrate used had a potency of 5000 and was furnished through the courtesy of Dr. R. J. Williams.

[5] Assays of the hydrolyzed yeast nucleic acid with *L. casei* showed it to be free of riboflavin, pantothen, biotin, niacin, and pyridoxine but appreciable amounts of folic acid were present.

of Norit A was added and adsorption allowed to continue for one hour at room temperature with constant stirring. This preparation was used in a concentration of one part in twenty parts of final medium.

*Niacin-free preparation (8L5C2)*

The use of copper precipitation, described above, was designed for the removal of nicotinic acid. While most of the niacin activity was removed by this method, as shown by the *L. casei* test, enough remained to warrant further treatment. Accordingly the filtrate from the copper precipitation was extracted with *n*-butanol for 96 hours in a continuous extraction apparatus (Wilson, Grauer, and Saier, 1940). It is known that nicotinamide is readily extracted with butanol, and after this treatment the extract was found to be entirely devoid of niacin activity, even when tested with *L. casei* in amounts four times greater than those used as a supplement for the ciliate. This preparation was used in a concentration of one part in twenty parts of final medium.

*Pyridoxine-free preparation (8L531L)*

This preparation was the least successful of any used. While it was possible to treat crude extracts and various filtrates in ways which would remove all pyridoxine activity for *L. casei,* it was usually found that the Factor II activity was also lowered to a point where the preparation was very inferior as a ciliate supplement. Therefore, the most satisfactory preparation, and the one finally used, was very low in Factor II activity, and the results obtained cannot be compared directly with those of the other vitamins tested. This preparation was made by exposing an alkaline lead acetate filtrate fraction (8L531), to direct illumination from a 300 watt electric bulb at a distance of 8 inches for a period of 72 hours. This method was used by Hochberg et al (1943) for pyridoxine destruction. Besides the destruction of appreciable amounts of the Factor II, another disadvantage of the technique was the excessive evaporation which took place during the treatment. It was necessary to add distilled water at frequent intervals to prevent the preparation from drying down. This preparation was used in a concentration of one part in ten parts of final medium.

*Assay procedure*

The base medium employed for the testing of the various preparations was the 16 amino acid mixture suggested by Hutchings and Peterson (1943). This was chosen in preference to the casein hydrolysate medium of Landy and Dicken (1942) because of the known composition of the former and the fact that lower blanks can be obtained. While the amino acid medium does not permit the production of as much acid by the bacteria it is very satisfactory for determining the presence or absence of a known vitamin.

Because of the scarcity of amino acids we have modified the usual procedure. The amino acid medium is made up for stock in double strength and the sugar, acetate, salts, purines and pyrimidine are added in double strength. For a test, this complete base medium is measured into 125 × 7 mm. Pyrex tubes in one ml. volumes. The material to be tested is added in appropriate amounts and a mixture

of the vitamins, minus the one for which the preparation is being tested, is added. The volume is then made up to 2 ml. with distilled water. Two controls were run with each test, one containing base medium and a complete set of supplements except for the vitamin under test. The second control contained the base medium plus the complete supplement and plus the Factor II preparation. The first served as a control on carry-over growth. The second was a control on the possible toxicity of the Factor II preparation. When titrations were made the figure from the first control was subtracted from the figure from the unknown preparation. Inasmuch as a small volume of medium was used it was found advantageous and more accurate to reduce the standard hydroxide to 0.05 N. The NaOH was standardized with 0.05 N oxalic acid, and the amount of acid produced after 96 hours of growth at 37° C. was titrated directly, using brom thymol blue as an indicator. The longer incubation period was used for maximum acid production, for in this way the test becomes more sensitive for traces of vitamins.

After many trials, the usual drop method of inoculation of *L. casei* was abandoned in favor of inoculating with a straight needle. This eliminates the necessity for washing the bacteria and blanks are just as low. The inocula were always made from yeast extract cultures which had incubated for 18–24 hours at 37° C.

While standard curves, using this method, have been made for all the vitamins studied the results obtained with our preparations do not permit quantitative statements as to amounts inasmuch as the tests were always made at very high levels and stimulatory materials in the Factor II preparations were invariably present. We were interested, moreover, first in the determination of the vitamin-free condition of our media, and second, in the biosynthesis of the vitamins by the ciliates. In the latter case, assays were employed on the medium before and after ciliate growth and the difference of acid production between the two compared directly.

It has been pointed out (see Cheldelin et al, 1942) that many of the B vitamins occur in a bound form in tissues and must be liberated by some means for satisfactory tests. There was the possibility that bound vitamins in the Factor II preparations might be available for the ciliate but not for *L. casei,* and these would invalidate any conclusions which were based on the vitamin-free nature of the preparation by the *L. casei* test. Enzymatic digestion was carried out on all the preparations, therefore, in order to test for the total vitamin content. Accordingly takadiastase and pepsin in quantities of one per cent each of the total solids of the preparation to be tested were used. The preparation was allowed to digest under toluene for 24 hours at 37° C. at pH 3.5. After steaming, the digest was added to the assay tubes, as described above, and a control of equivalent amounts of the enzymes added to parallel tubes. This latter control is obviously necessary as the enzymes are not vitamin-free. Data on the results of assays of the Factor II preparations used are presented in Table II.

*Ciliate cultures*

It was the usual practice, when testing for the effects of one of the known vitamins, to grow the ciliate through at least three serial tube transplants in the medium containing the vitamin being investigated, paralleled with the same medium minus the vitamin. Transplants were made at 72 hour intervals with a bacteriological loop delivering approximately 0.005 ml. of fluid. All incubation was at

25° C. Growth rate was followed by inoculating appropriate amounts of third transplant ciliates (36 hours old) into like media in culture flasks (Kidder, 1941). After inoculation of the flasks (as near 100/ml. as possible) samples were drawn and the initial inoculum determined. Growth thereafter was ascertained by sampling at intervals until the termination of the experiment. In all cases the flask series were repeated at least once and the figures averaged.

TABLE II

*Assay of Factor II Preparations with Lactobacillus casei 912*

| No. | Additions | Vitamin omitted from base medium | | | | | |
|---|---|---|---|---|---|---|---|
| | | Riboflavin | Pantothen | Biotin | Nicotinamide | Pyridoxine | Folic acid |
| 1 | None | 0.05 | 0.00 | 0.05 | 0.49 | 0.12 | 0.62 |
| 2 | Enzyme preparation | 0.13 | 0.38 | 0.17 | 0.75 | 0.10 | 0.90 |
| 3 | 8L531 | 0.05 | 3.79 | 2.88 | 4.72 | 3.86 | 0.00 |
| 4 | 8L531H | 0.03 | 0.12 | 2.79 | 4.80 | 2.60 | 0.00 |
| 5 | 8L5C1 | 0.00 | 1.53 | 0.07 | 0.22 | 3.10 | 4.36 |
| 6 | 8L5C2 | 3.42 | 0.87 | 2.93 | 0.00 | 3.65 | 4.65 |
| 7 | 8L531L | 0.00 | 3.74 | 3.00 | 4.51 | 0.09 | 0.00 |

Figures represent ml. of 0.05 N acid per culture (2 ml.). All figures corrected for uninoculated blanks. Line 2 corrected for carry-over growth (Line 1). Lines 3–7 corrected for vitamin content of enzyme preparation (Line 2).

One obvious objection to the flask technique is the possibility of introducing the vitamin being investigated from the rubber vaccine tip used in the sampling port. This possibility was diminished by boiling the vaccine tips for one hour previous to setting up the flasks. As a check on the tips uninoculated flasks were manipulated in the same manner as the experimental cultures and the samples tested with *L. casei* for the vitamin being studied. In no case were these detectable amounts of the vitamins present. Sampling needles were made chemically clean as well as sterile before use.

Growth rate during the logarithmic phase was calculated by the formula $g = \frac{t \log 2}{\log b - \log a}$ where $t$ = the time in hours during which the population has been increasing exponentially, $a$ =the number of cells per unit volume at the beginning, and $b$ = the number of cells at the end of time, $t$.

## RESULTS

After obtaining Factor II preparations which were free of the vitamins to be studied, preliminary experiments were set up to determine which vitamins, if any, were essential growth factors for *Tetrahymena geleii* W. Accordingly serial transplants were made in the appropriate media, one set with the vitamin present, and the other with the vitamin omitted. It was immediately apparent that the ciliate lacked all ability to synthesize folic acid (Kidder, 1945) but the absence of none of the other vitamins did more than lower the growth rate and the yield. Growth in

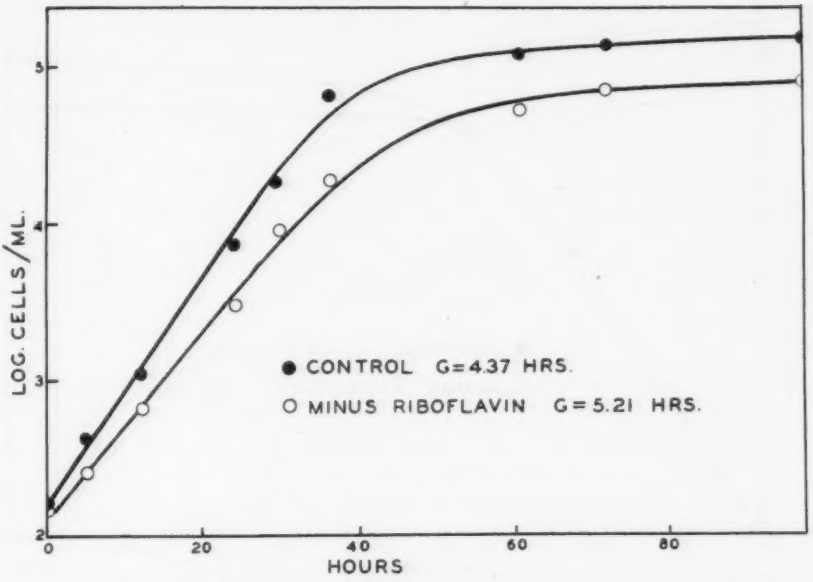


FIGURE 1. Effect of the omission of riboflavin. Factor II preparation used was 8L531. Average of two separate experiments.

TABLE III

*Summary of Growth Data*

| Medium | Generation time in hours | Population per ml. at end of log. phase | Population per ml. at 96 hours |
|---|---|---|---|
| Control | 4.37 | 58,000 | 164,000 |
| Minus riboflavin | 5.21 | 19,000 | 67,000 |
| Control | 4.57 | 32,000 | 90,000 |
| Minus pantothen | 4.60 | 34,500 | 41,000 |
| Control | 4.32 | 45,500 | 152,000 |
| Minus biotin | 5.01 | 15,000 | 96,000 |
| Control | 4.17 | 49,000 | 170,000 |
| Minus nicotinamide | 8.40 | 7,500 | 79,000 |

the sixth serial transplant was possible for all series except that lacking exogenous folic acid.

In order to gain quantitative information regarding the stimulatory effect that was apparent in the tube cultures, growth flasks were inoculated from third transplant tubes and the growth followed by frequent sampling. In the case of pyridoxine, however, the flask cultures were omitted, as the Factor II preparation necessarily used was relatively inactive and the growth was erratic, even when pyridoxine was

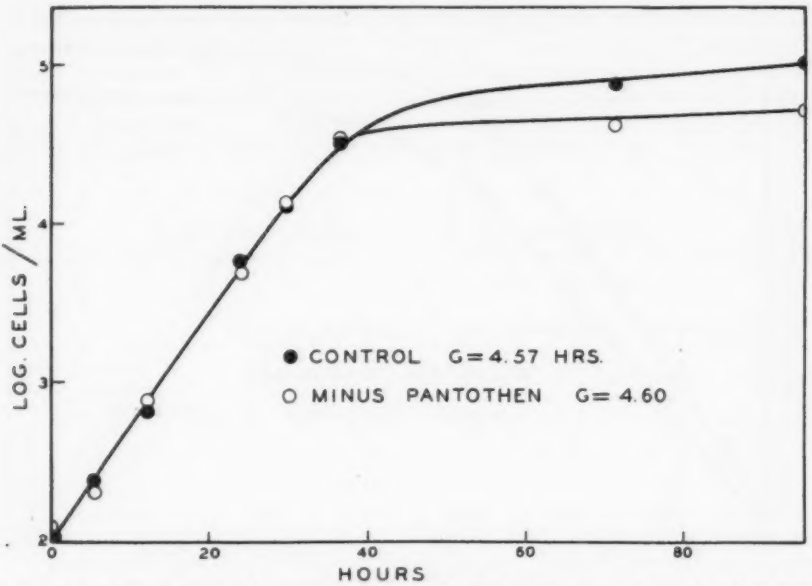


FIGURE 2. Effect of the omission of pantothenic acid. Factor II preparation used with 8L531L. Average of two experiments.

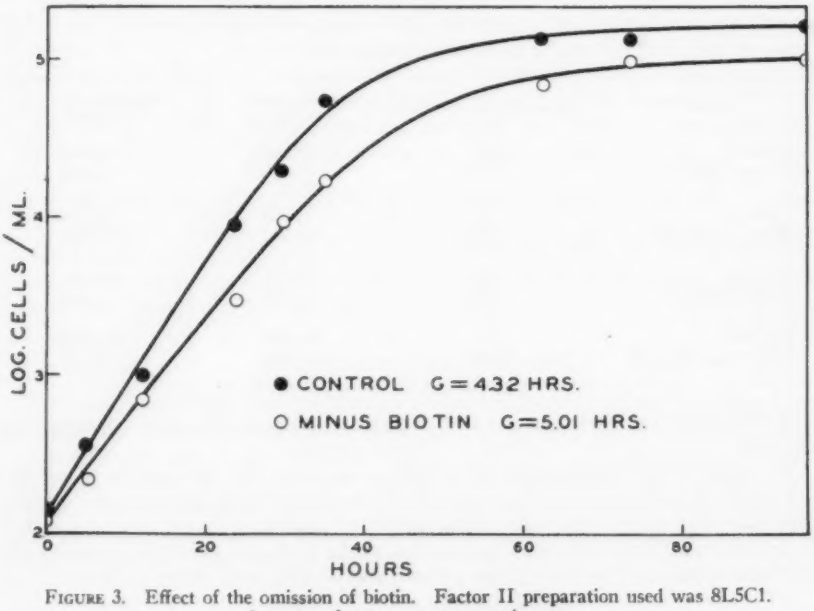


FIGURE 3. Effect of the omission of biotin. Factor II preparation used was 8L5C1. Average of two separate experiments.

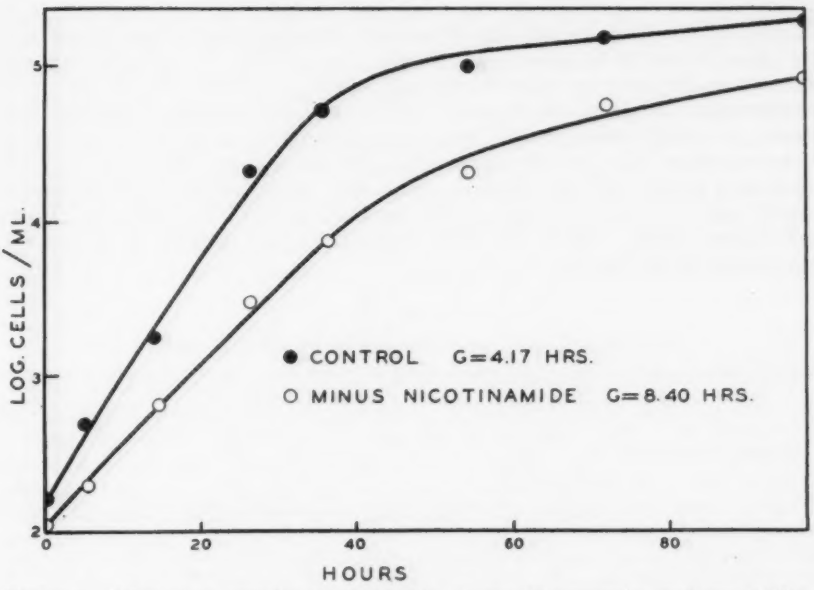


FIGURE 4. Effect of the omission of nicotinamide. Factor II preparation used was 8L5C2. Average of three separate experiments.

present. While qualitative data are lacking for the pyridixine series, nevertheless we can say from the serial tube transplants that this vitamin appears to be only stimulatory for *T. geleii* W.

The omission of riboflavin from the medium resulted in slower growth during the exponential period. Thus the generation time was raised from 4.47 hours in the control flasks to 5.21 hours. The maximum yields were reduced to less than half of those in the control flasks (Fig. 1; Table III).

The ciliates appear to synthesize pantothen at a rate which equals the demands for rapid growth, as judged by the almost identical growth rates in the pantothen-containing and the pantothen-free media (Fig. 2; Table III). In all cases, however, the maximum yield was significantly lower in the pantothen-free cultures.

A comparison of the growth curves, generation times and yields for biotin-free and riboflavin-free media (Figs. 1, 3; Table III) shows remarkable similarity. The rate of synthesis of biotin by the ciliates appears to be low, indicating the stimulatory status of this vitamin. We possess added data on biotin substantiating its non-essential nature for *T. geleii* W. Early in this series of investigations the effect of raw egg white and avidin concentrates were studied as a means of determining whether or not the ciliate required biotin. Egg white was taken aseptically and added to tubes containing 5 ml. of one per cent proteose-peptone, each tube receiving 0.1 ml. According to Eakin, Snell, and Williams (1941), this amount of egg white is enough to inactivate 0.05 micrograms of biotin. The analysis of proteose-peptone made by Stokes, Gunness, and Foster (1944) shows that one gram contains

0.2 micrograms of biotin, hence our tubes each contained 0.01 micrograms of the vitamin. The amount of raw egg white used, therefore, was enough to inactivate five times more biotin than was present. Indefinitely transplantable growth occurred in the proteose-peptone plus egg white. Likewise, the use of avidin concentrates in quantities far in excess of that needed to inactivate all of the biotin present, produced similar results. In this case the avidin was allowed to act on the proteose-peptone, the peptone removed as the diffusate in dialysis, the peptone being used as the medium. Similar results were obtained with proteose-peptone treated with $H_2O_2$ in a manner similar to that described by Garnjobst, Tatum, and Taylor (1943). While it is clear that biotin is not required by *T. geleii* W this vitamin is stimulatory.

TABLE IV

*Assay Data (L. casei) Before and After the Growth of T. Geleii W*

| Additions for assay (1:10) | Factor II preparation | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 8L531 | | 8L531H | | 8L5C1 | | 8L5C2 | | 8L531L | |
| | Plus riboflavin | Minus riboflavin | Plus pantothen | Minus pantothen | Plus biotin | Minus biotin | Plus nicotinamide | Minus nicotinamide | Plus pyridoxine | Minus pyridoxine |
| Before inoculation | 4.78 | 0.07 | 3.90 | 0.17 | 3.92 | 0.08 | 3.88 | 0.00 | 3.61 | 0.16 |
| After 72 hr. ciliate growth. Medium plus cells | 4.60 | 1.18 | 3.94 | 1.71 | 3.90 | 1.56 | 3.80 | 0.21 | 3.48 | 0.22 |
| Supernatant of 72 hr. ciliate culture | 4.82 | 0.06 | 4.13 | 0.10 | 3.86 | 0.10 | 3.71 | 0.16 | 3.52 | 0.11 |
| Washed ciliates from 72 hr. culture | 4.80 | 1.07 | 3.94 | 1.64 | 3.91 | 1.05 | 3.75 | 0.25 | 3.61 | 0.15 |

Figures represent ml. of 0.05 N acid per culture (2 ml.). All figures corrected for uninoculated blanks and for carry-over growth.

While *T. geleii* W can be transplanted indefinitely in the absence of exogenous nictotinamide this vitamin (or nicotinic acid) is an active stimulant. The generation time is doubled when the ciliate is grown in niacin-free medium as compared to that in the nicotinamide-containing control (Fig. 4; Table III). While the population density at 96 hours is less than one-half that of the control (which is similar to the cases of riboflavin, biotin, and pantothen), the population at the end of the logarithmic phase is extremely low (approximately 7000/ml.).

It was of interest to determine whether or not *T. geleii* W would synthesize amounts of the vitamins which could be detected with the assay methods used. Accordingly the five types of media used above were set up for serial transplants and an aliquot of each was assayed with *L. casei*. After the ciliates had grown for 72 hours in the third transplants, assays were again made for the various vitamins. These assays were of three different types. One was on the whole medium (medium plus cells); one, on the supernatant fluid following centrifugation after chilling (Kidder, Stuart, McGann and Dewey, 1945), and the third was on washed cells equivalent to the concentrations found in the whole medium. The samples to be tested were added to the *L. casei* base medium and sterilized by auto-

claving. The results of these experiments are given in Table IV. Appreciable amounts of riboflavin, pantothen, and biotin are synthesized by the ciliates. Increases in amounts of niacin are so small that they probably lack significance and there appears to be no increase in pyridoxine. It must be remembered, however, that the growth in the niacin-free medium is less at 72 hours than in the riboflavin-, pantothen-, or biotin-free media, while the maximum population reached in the pyridoxine-free medium never exceeded 20,000 ciliates per ml. The amounts of the vitamins detected represent minimums, as no attempt was made to release any which may have been bound (except by autoclaving). It is to be noted that all vitamins which were synthesized remained in the cells. This was also found to be true in the case of the biosynthesis of thiamine by *T. geleii* W (Kidder and Dewey, 1942).

## Discussion

Due to the various treatments necessary for the removal of vitamins none of the Factor II preparations used in this study produced as high yields as had been previously obtained (Kidder and Dewey, 1945d; Kidder, 1945). While the riboflavin-free preparation was essentially the same as had been used for the study of purines and pyrimidines and of folic acid, variations in potency of Factor II activity were evident. This is due almost entirely to the degree of adsorption on the activated charcoal. Slight variations of temperature appear to effect the degree to which Factor II is lost, so that a critical balance is found between the complete removal of the vitamins and the loss of Factor II activity. In this study the emphasis was placed on the vitamin removal at a sacrifice of yield.

The findings of Hall and Cosgrove (1944) on the importance of riboflavin for their strain of *Tetrahymena geleii* does not seem inconsistent with the present observations. They state that heat—and alkali-treated casein did not support growth unless supplemented with thiamine, and even then poorly. The addition of riboflavin together with the thiamine, however, permitted as good growth as did the casein medium before heating. There can be no doubt as to the stimulatory effect of riboflavin, and it is altogether possible that it may function as a detoxifying agent as well. The detoxifying action of thiamine has been suggested previously in this connection (Kidder and Dewey, 1944).

In addition to the vitamins which have already been investigated for *T. geleii* W there remain at least three of the commonly recognized ones about which little is known. These are *p*-aminobenzoic acid, inositol and choline. As yet we have not had the opportunity to test for the last two, but preliminary work has been started on the first. The commonly employed technique of adding sulfonamides to the medium has indicated that this ciliate requires excessive amounts of the inhibitor to effect growth. The inhibition to growth at these high levels is not completely reversed with *p*-aminobenzoic acid, and the evidence indicates that purines are also involved. This study awaits completion and will be reported at a later date, but it appears that *T. geleii* W may be independent of an exogenous supply of *p*-aminobenzoic acid.

The only other protozoan of animal nature about which there appears to be critical data regarding the requirements of the vitamins studied here is *Colpoda duodenaria* (Tatum, Garnjobst, and Taylor, 1942; Garnjobst, Tatum and Taylor, 1943).

Colpoda requires large amounts of thiamine, pantothen, riboflavin, nicotinamide, and pyridoxine. It does not require *p*-aminobenzoic acid, biotin, or inositol, while the status of choline and folic acid is still unknown. Moreover, Colpoda was shown (Garnjobst, Tatum, and Taylor, 1943) by the *Neurospora* test of Tatum and Beadle (1942) to either release bound biotin from the bacterial "plasmoptyzate" used or to synthesize this vitamin. This biotin appeared in the medium, however, and in this way differs from the condition found with *T. geleii* W where all of the vitamins arising by biosynthesis appear to bound in the cell protoplasm.

The biochemical investigations of *Tetrahymena geleii* W which have so far been completed permit a fairly complete view of its synthetic abilities. Added carbon sources appear to be unnecessary except as they may perform a sparing action on the amino acids. Inorganic salts certainly are essential (Hall and Cosgrove, 1944; Kidder and Dewey, 1944) although the question of which elements need to be included is yet to be determined. The commonly employed inorganic salts usually accepted as being physiologically important satisfy the ciliate requirements. Nine amino acids are to be classed as indispensable for this strain (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophane, and valine) while arginine is synthesized at so low a rate that its inclusion becomes obligatory. Serine is extremely stimulatory, but its place may be taken by others of the dispensable amino acids (Kidder and Dewey, 1945c). The list of essential growth factors for this strain is not long. Purines (most effectively guanine) and pyrimidines (cytidylic acid and/or uracil) must be supplied in rather large amounts (Kidder and Dewey, 1945d), and folic acid must be present in amounts in excess of that required for most of the folic acid-requiring bacteria (Kidder, 1945). Factor II must be supplied. This substance (or substances) is still chemically undefined, but it possesses similarities to the "streptogenin" of Woolley (1941) and Sprince and Woolley (1944).

Biosynthesis of riboflavin, pantothen, and biotin can be accomplished by *T. geleii* W. Indefinitely transplantable growth results without exogenous thiamine (Kidder and Dewey, 1942; 1944; 1945b), riboflavin, pantothen, biotin, niacin, or pyridoxine. There is some evidence to indicate that *p*-aminobenzoic acid may not be essential, and the status of inositol and choline is still unknown.

For practical purposes it is always of advantage to include any substances of a stimulatory nature. The absence of any one of the stimulatory substances (thiamine, riboflavin, pantothen, biotin, niacin, pyridoxine) will become a limiting factor, decreasing the growth rate or the maximum yield or the longevity of the culture (Johnson and Baker, 1942; Hall, 1944). The stimulatory vitamins should be included in the culture medium of this ciliate when maximum growth is desired.

## Summary

1. It has been possible to prepare media for the growth of *Tetrahymena geleii* W which are free of riboflavin, pantothen, biotin, niacin, and pyridoxine, as determined by the *Lactobacillus casei* test.

2. *T. geleii* W is not dependent on an exogenous source of any one of the above vitamins. Omission of any one, however, reduces the maximum yield and, with the single exception of pantothen, the growth rate.

3. Biosynthesis of appreciable amounts of riboflavin, pantothen, and biotin occurs. These vitamins are found bound in the cell protoplasm. No significant increases of pyridoxine by biosynthesis were found.

4. The five vitamins listed are not essential growth factors for *T. geleii* W but are stimulatory factors, and as such should be included in the medium for optimum growth.

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# THE STRUCTURE OF MEIOTIC CHROMOSOMES IN THE GRASSHOPPER AND ITS BEARING ON THE NATURE OF "CHROMOMERES" AND "LAMP-BRUSH CHROMOSOMES"

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The nature of the gene is one of the fundamental problems in modern biology. Since the genes are located in the chromosomes, the structure, chemistry, and metabolism of the chromosomes are of special significance for the understanding of the gene and gene action. The prevalent interpretation of chromosome structure has developed as a kind of compromise between two originally opposed views, the "chromomere hypothesis" of Balbiani, Pfitzner, and Strasburger and the "chromonema hypothesis" of Baranetzky, Bonnevie, and Vejdovsky.[2] According to the "chromomere hypothesis," the chromosome consists of a series of small beads or discs strung together. During prophase they approach each other, fuse into larger complexes, and finally disappear in the thick rod-shaped metaphase chromosomes. For the "chromonema hypothesis" on the other hand, the fundamental unit of the chromosome is a coiled thread, tightly wound in a helix at metaphase and more or less uncoiled during interphase. Both chromomeres and spirals were discovered about the same time (Balibiani, 1876; Pfitzner, 1882; Baranetzky, 1880). Yet more and more structures first described as "chromomeres" have turned out to be coils and today the "chromomere" is in full retreat into the submicroscopic level. Strasburger's "chromomeres" in Tradescantia pollen mother cells had been clearly shown to be spirals by Baranetzky (1880); Pfitzner's "granules" in somatic prophases of the salamander were resolved into coils by Schneider (1910) and by Lee (1921), who concluded that all "chromomeres" are in reality turns in the helix. The modern view which is accepted by most cytologists today and is based mainly on Heitz (1935), holds that the true "chromomeres" (Belling's ultimate chromomeres) can only be seen in the prophase of meiosis (leptotene) and in the curious giant chromosomes of dipteran larvae, where the chromonemata are assumed to be completely uncoiled. According to this view (Reuter, 1930; Heitz, 1935; Darlington, 1937; White, 1937; Geitler, 1938; Koltzoff, 1938; Kuwada, 1939; Nebel, 1939; Huskins, 1941, 1942; Straub, 1943) the chromonema consists of chromomeres of different but constant size, rich in nucleic acid, connected by protein fibrils. The chromomeres bear the genes, they reproduce as specific units and they synapse in meiotic prophase. They are the visible expression of the linear arrangement of the genes.

[1] Part of the work for this paper was done in the Department of Biology, Johns Hopkins University.

[2] The "vacuolization hypothesis" of Grégoire and his school, denying both chromomeres and chromonemata, has been thoroughly disproved by the work of the last twenty years and need not be discussed here.

Yet even in leptotene chromosomes the "chromomeres" were found to be coils by several authors. They were first described as such in Tradescantia by Kaufmann (1931), who nevertheless accepted the "chromomere" interpretation for other plants and animals (Kaufmann, 1936). Koshy (1934, 1937) found the leptotene chromosome to be coiled in Allium and Aloe, Naithani (1937) in Hyacinthus. Smith (1932) suggested that the beadlike appearance of the leptotene in Galtonia might be due to twists in the chromonema and Hoare (1934) noted that the zygotene threads give the impression of two tightly coiled chromonemata. Kuwada (1939) pointed out that sharp turns in the coils might easily be mistaken for "chromomeres." In Tradescantia, Swanson (1943) found no "chromomeres" which could not be resolved into coils, and he suggested that a chromomere pattern such as that in maize might be due to differential spiralization.

Yet most recent discussions on the gene and chromosome structure cling tenaciously to the belief that "chromomeres" are real (e.g., Schultz, 1944). The main evidence usually presented, besides the salivary chromosomes of dipteran larvae, is the observations of Wenrich (1916), Lewis and Robertson (1916), and Chambers (1924) on the large chromosomes in grasshopper spermatocytes. To re-examine this evidence is the purpose of the present investigation.

## Material and Methods

Spermatocytes of *Chorthippus curtipennis, Chorthophaga viridifasciata, Dissosteira carolina, Melanoplus femur-rubrum, Arphia* sp., *Hippiscus* sp., and *Orphulella* sp. were studied in sections (fixation: B 15 and Sanfelice, stain: Feulgen), and aceto-orcein smears. For the detailed study of leptotene chromosomes sections stained with Feulgen were found to be more reliable than smears. To uncoil chromosomes, testes were submersed for one-two hours in $2 \cdot 10^{-3}$ M KCN in Bělař solution (Bělař, 1929) before smearing (Oura, 1936). The optics used consisted of a Zeiss aplantic condenser N.A. 1.4, Zeiss 2 mm. objective N.A. 1.4 and 15× ocular. The photographs (except Figure 12) were taken with the same optics and a Bausch and Lomb photomicrographic camera type H. The stereoscopic photographs were made by shifting the substage diaphragm maximally to the left and right respectively for the two exposures. [3]

## The Structure of Leptotene Chromosomes

On casual examination the slender, irregularly twisted chromosomes at leptotene have a beaded appearance as has been so often described in the literature (for a review see Reuter, 1930). A detailed study with the best optics and a delicate use of the fine adjustment screw of the microscope, however, resolves the beads or "chromomeres" into turns of a narrowly pitched coil [4] (Figures 1, 6a, and 13). With Feulgen the chromosome stains evenly throughout its length and there are no Feulgen-negative "interchromomeric fibrils." This uniform nature of the

[3] I wish to thank Mr. John Spurbeck, Dept. of Biology, Johns Hopkins University, for help with the photomicrographs.

[4] Mr. L. Vanderlyn, Dept. of Zoology, University of Pennsylvania, informs me that he has come independently to the conclusion that the "chromomeres" are in reality gyres in the chromonemata. In a forthcoming paper he will trace the origin of these from the unpacking coils of the preleptotene in *Podisma alpina*.

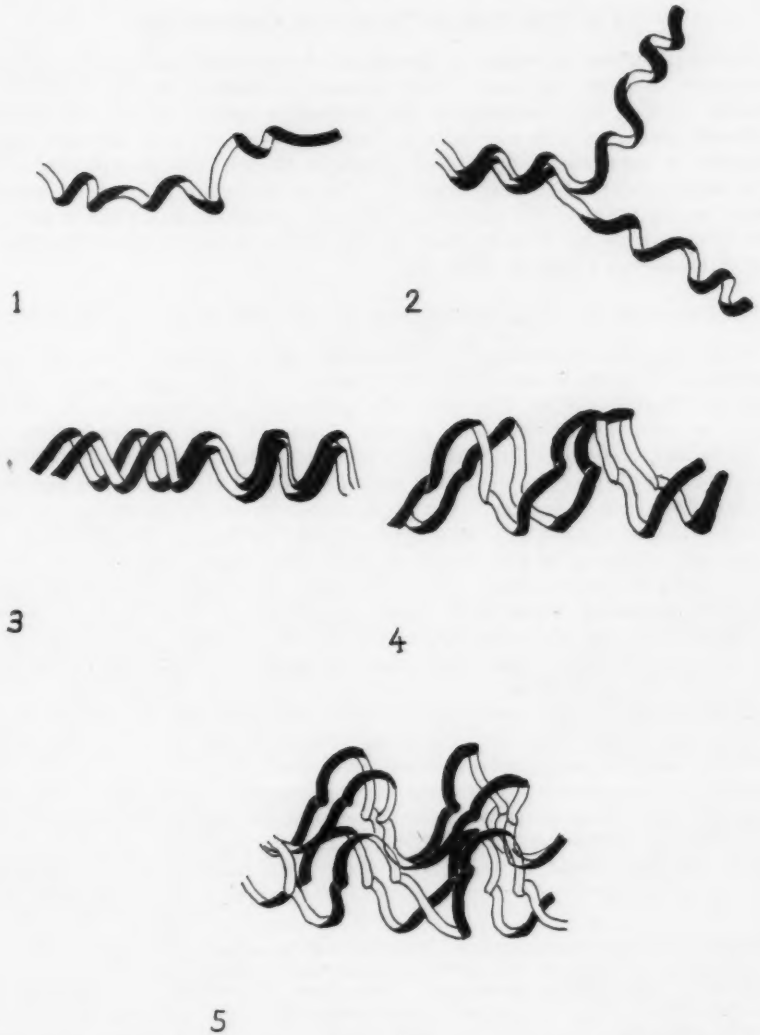
leptotene chromosomes can best be seen in well fixed sections. A chromosome, followed with the fine adjustment as it winds itself through the nucleus, is seen to be a thread of uniform thickness thrown into a tight, irregular helix. The narrow turns of this coil where the chromosome overlaps itself, appear as "chromo meres." The gyres can vary in width and may be unevenly spaced (see Figure 13). This can give the impression of different sized chromomeres. The width of the thread and the tightness of the helix are characteristic for each species of grasshopper studied. In aceto-orcein smears, when the chromosome has been under shear or pressure, an apparent chromomeric structure is more pronounced. This is due to the wax-like consistency of the chromosome which causes its gyres to fuse or be pulled out and otherwise distorted. Chromosomes, in which the coils can be clearly seen, can easily be transformed into the classical string of beads simply by exerting pressure on the coverslip and smearing them out. It is interesting to note in this connection that Belling (1931) emphasized that chromomeres are not clear in sections and that one has to use smears to make them visible.

When does that tight irregular coil of the leptotene chromosome originate? Is there any stage when the chromonemata are completely stretched out and without any signs of coiling? In all the grasshoppers studied no chromosome was found that did not show some degree of coiling. Furthermore, the characteristic coil of the leptotene chromosome is already present in the interphase and unravelling stage of preleptotene. We must assume that the leptotene spiral originates in the interphase or telophase of the preceding division. This origin of a prophase helix in the preceding telophase has been demonstrated by Sparrow (1942) in the microspore division in Tradescantia. The chromosome of the unravelling stage is thus doubly coiled (Figure 7). It shows the wide gyres of the previous metaphase relaxing into the relic coils of leptotene and the small tight helix which is destined to enlarge during pachytene and become the major coil of the first meiotic metaphase chromosome. This structure of the preleptotene chromosome was indicated clearly in McClung's figures for *Mecostethus lineatus* (esp. Figure 43, McClung, 1927). The heteropycnotic X chromosome in the prophase of grasshopper spermatocytes, which does not unwind in preleptotene and is thus comparable to the preleptotene autosomes in structure, similarly discloses a small tight helix and a wide irregular coil as Coleman (1943) has demonstrated.

Since the preleptotene chromosome consists of at least two chromonemata the leptotene chromosome also must be double (Robertson, 1931). The split between the chromatids can sometimes be discerned, especially in the turns of the coil, but usually the sister strands are closely appressed. They seem to form a plectonemic spiral, though this could not be determined with certainty.

## The Structure of Zygotene Chromosomes

The pairing of homologous chromosomes at zygotene thus takes place between two coiled structures. The gyres of the two chromosomes fit into each other and become more or less closely appressed (Figures 2 and 6b). The bivalent now forms a paranemic coil. Just as the gyres in leptotene were mistaken for "chromomeres," so the gyres of the parallel coil in the bivalent were thought to be paired "chromomeres."




FIGURES 1–5. Diagrammatic representation of chromosome structure during meiotic prophase of the grasshopper.

FIGURE 1. Leptotene.

FIGURE 2. Zygotene.

FIGURE 3. Pachytene. The homologues can be either slightly separated or closely appressed.

FIGURE 4. Later pachytene. Appearance of the minor coil.

FIGURE 5. Diplotene. The chromonemata have separated laterally. This represents in essence also the structure of "lamp-brush chromosomes."

### The Structure of Pachytene Chromosomes

During pachytene the helices of the paired chromosomes increase in width and the number of gyres decreases. This process is identical to that described by Swanson (1942a) for Tradescantia (despiralization cycle). If the chromosomes are closely appressed only one helix is visible. When the coils separate slightly a reticular or vacuolated appearance is produced, though often two parallel helices can be clearly discerned (Figures 3 and 8). In late pachytene an irregular waviness appears on the gyres of the pachytene coil; this sometimes looks like a very fine spiral of narrow pitch. It most likely corresponds to the minor spiral described in plant chromosomes (Figures 4 and 9).

### The Structure of the Chromosomes During Diplotene and Diakinesis

In this stage the chromosomes are most difficult to analyze. They are usually described in the literature as diffuse, having fuzzy or woolly fringes (see for instance Nebel and Ruttle, 1937). The better the general fixation seems to be, the less distinct or sharp the chromosomes appear. However, after submersing the cells for one to two hours in $2 \cdot 10^{-3}$ M KCN in Bělař solution and staining in aceto-orcein, the structure of the diakinesis chromosome and the reason for its woolly appearance becomes quite clear. The lateral separation of the chromonemata which had already begun in pachytene has progressed much further, so that their gyres now overlap only within a narrow central region. This region appears as a beaded darker core of the chromosome. The gyres of the major coil of the chromonemata form loops projecting beyond this central core (Figures 5 and 14). It is these loops of the individual chromonemata which give the chromosome its hairy appearance. If the separation of the coiled threads is great the chromosome looks like a dark, beaded rod with loops or hairs at regular intervals (Figure 14a). When the lateral shifting is less the chromosome gives the impression of a double beaded rod, the loops or hairs now of course being shorter (Figure 14b). These appearances can easily be explained on a model of four simultaneously coiled wires. Sometimes one or more irregular turns of the minor coil can be seen on the loops.

In this stage there is further evidence against the reality of "chromomeres." If the apparent thickenings in the leptotene chromosome were constant units of definite size, they should be visible also in the loops of the diplotene chromatids.

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Plate I

Figure 6. Chorthophaga, zygotene. Pretreated with ammonia vapor. Aceto-orcein smear. Note the coil of the univalent at *a* and the paranemic helix of the bivalent at *b*.

Figure 7. Chorthophaga, preleptotene. Aceto-orcein smear. Irregular "major coil" in the process of unravelling. The narrowly pitched helix ("minor coil") corresponds to the leptotene spiral (arrows).

Figure 8. Chorthippus, early pachytene. Section. Fixed with Sanfelice and stained with Feulgen.

Figure 9. Hippiscus, late pachytene. Section. Fixed with Sanfelice and stained with Feulgen.

Figures 10 and 11. Orphulella, pachytene. Pretreated for 2 hours in KCN. Aceto-orcein smear. The heterochromatic knobs have been resolved into coils (arrows).

Figure 12. Fragment of a "lamp-brush chromosome" from a frog oöcyte. Aceto-orcein smear. Note the loops of the major coil and the minor coil (arrows). Zeiss 3 mm. objective, 15 × ocular.

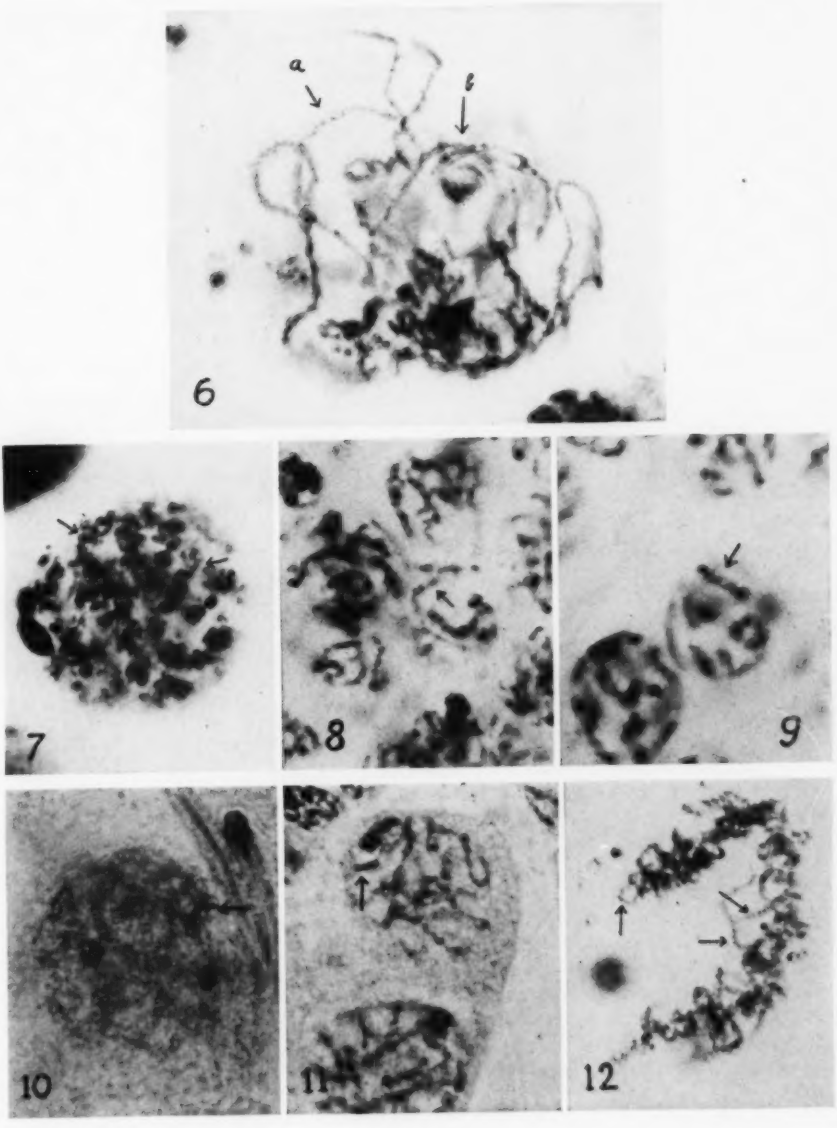



PLATE I

These chromatids, however, never show any beaded structure. The despiralization already noted in pachytene has continued and has resulted in an increase in width and decrease in the number of gyres with a consequent shortening and thickening of the chromosome.

### The Structure of Metaphase Chromosomes

At the end of diakinesis the gyres of the chromatids become more closely spaced along the chromosome axis, leading to a further shortening of the chromosome and a fusion of the "chromatic coating" (Ris, 1942) of the individual chromatids, so that a uniformly staining body results. The chromatids retain their lateral separation, causing what is sometimes observed as a reticulate or vacuolated appearance of the metaphase chromosomes.

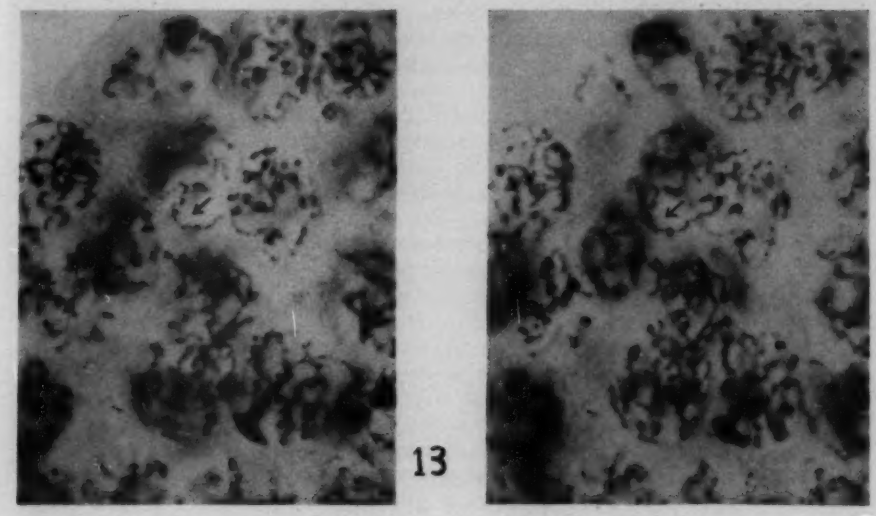
### The Nature of the Heteropycnotic Regions in Orphulella

During meiotic prophase the chromosomes of Orphulella carry small, knob-like, darkly staining bodies, especially at their ends. These structures resemble the large "chromomeres" described by Wenrich (1916) in Phrynotettix. Treatment with KCN for 3 hours causes a loosening of the chromosome helix and shows that these knobs are tightly coiled regions of the chromosome (Figures 10 and 11). It is evident that the different appearance of such heteropycnotic regions in meiotic chromosomes is mainly due to differential coiling of the chromonemata as has been shown for the X chromosome by Coleman (1943). Similarly Wilson and Boothroyd (1944) have demonstrated that heterochromatic differentiations after cold treatment are the result of differential coiling.

## Discussion

### *Chromomeres*

The synthesis of cytology and genetics in the chromosome theory of inheritance has had a stimulating effect on the investigation of chromosomes. Yet the knowledge of the intimate structure of the chromosome has been retarded rather than furthered by the influence of genetics. The constant desire to find visual expression of the linear order of genes has led to the perpetuation of misinterpretations of the microscopic image. Indeed cytogenetics has established beyond doubt the longitudinal differentiation of chromosomes, but it is not justifiable to conclude that the units of this differentiation are microscopically visible particles. Thus observations which did not agree with the "chromomere" hypothesis tended to be ignored. The extensive literature on the subject (see Reuter, 1930) shows the widespread acceptance as well as the great versatility of the chromomere concept. Almost any expression of unevenness along the chromosome was at one time or other called "chromomere." The first pictures of "chromomeres" were published by Balbiani (1876) and Pfitzner (1882). Both described prophase and metaphase chromosomes in somatic cells. Today there can be no doubt that they saw the gyres of the somatic helix (Schneider, 1910; Lee, 1921; Creighton, 1938). Strasburger (1882) and Farmer and Shove (1905) described disc-like "chromomeres" in meiotic metaphase chromosomes of Tradescantia. We know now that they mistook the gyres of the major coil for discs. Quite often chromocenters in



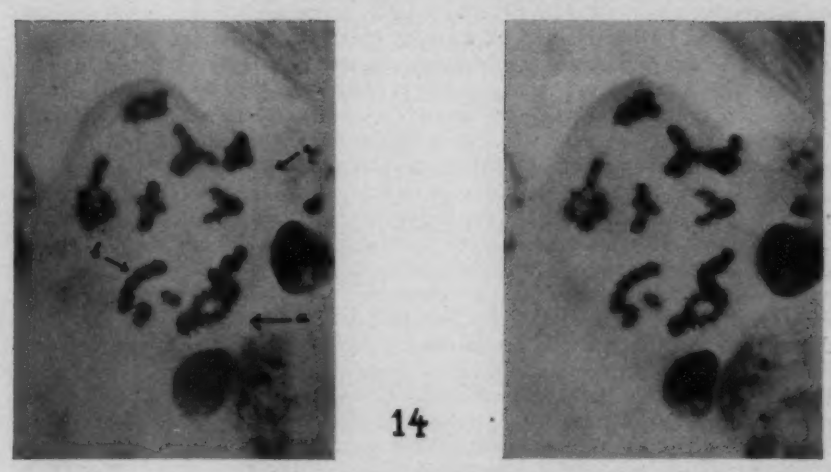




PLATE II

FIGURE 13. Stereophotomicrograph, Chorthippus leptotene. Section. Fixed with Sanfelice and stained with Feulgen and Iron hematoxylin. Note the coiled leptotene chromosomes (arrow).

FIGURE 14. Stereophotomicrograph, Hippiscus diakinesis. Pretreated with KCN. Aceto-orcein smear. Note the loops of the major coil which give the chromosomes at this stage the fuzzy appearance.

interphase nuclei and heteropycnotic regions on the chromosome, such as found in the X chromosome of *Notonecta indica* (Browne, 1916), were called "chromomeres" (cf. Heitz, 1929). Shinke (1937) and Coleman (1940, 1941) have shown that such heteropycnotic regions are parts of the chromonema which remain tightly coiled or become precociously coiled. This could be confirmed in the present paper for the "knobs" of the meiotic chromosomes of Orphulella. Thus, one more "chromomere" was reduced to chromonematic coiling. There remained the "ultimate chromomere" of Belling (1928), the only bona fide "chromomere" according to most modern cytologists. This "chromomere" can only be seen in meiotic prophase and in salivary chromosomes of dipteran larvae, where the chromonemata are assumed to be maximally stretched. Let us examine point for point the evidence which is given for the reality of these "chromomeres" (see reviews cited in introduction).

(*a*) *"The chromomeres are seen in living cells and cannot be artefacts."* Bělař (1928) described "chromomeres" in living spermatocytes of the grasshopper. An analysis of his figure shows that he did not see chromomeres but the coils of diakinesis chromosomes. Lewis and Robertson (1916) and Chambers (1924) found "chromomeres" in the leptotene of living grasshopper spermatocytes. This may show that the structures observed are not fixation artefacts, but it certainly is easier to misinterpret narrow coils as granules in unstained cells where the chromosomes are hardly visible, than in well stained preparations. Yet there is a very interesting observation by Chambers (1924, page 270) which seems to have been overlooked by himself as well as most reviewers of chromosome structure. He writes: "If one of the early prophase chromosomes with ragged granular outlines be seized with a needle and rapidly pulled across the field so as to stretch it, the granules disappear and the whole substance becomes homogeneous." So Chamber's microdissection study does not support the "chromomere" hypothesis, but rather the assumption of a uniform but coiled leptotene chromosome.

(*b*) *"The chromomeres have specific and constant sizes and form a definite pattern."* The classical examples are Dendrocoelum (Gelei, 1921) and Phrynotettix (Wenrich, 1916). The observed patterns in these and other forms are an expression of the longitudinal differentiation of the chromosome. This differentiation is real. But the nature of this differentiation now turns out to be differential coiling and not a sequence of discrete bodies of different sizes. The large "chromomeres" in Phrynotettix are heterochromatic regions along the chromosome similar to those found in certain plant chromosomes and those described for Orphulella in this paper. In *Veltheimia viridifolia* Coleman (1940) could show that such heterochromatic regions are closely coiled sections of the chromonema. They correspond in structure to the differential segment in Rhoeo (Coleman, 1941) and the chromocenters in various animals and plants (Shinke, 1937). The knobs in maize are most probably of a similar nature.

(*c*) *"The chromomeres of homologous chromosomes pair specifically at zygotene."* Just as the turns in the spiral give the impression of "chromomeres" at leptotene, the paranemic spiral of the paired bivalent simulates a row of paired granules. Since homologous regions of the chromosomes pair, it is evident that heterochromatic sections will come to lie side by side in the pachytene chromosomes.

(*d*) *"The number of chromomeres in leptotene corresponds approximately to the number of genes in Lilium* (Belling, 1928). *In salivary chromosomes the*

*bands, which correspond to the leptotene chromomeres, were shown to be closely associated with certain genes* (Muller and Prokofyeva, 1935)."

Belling's estimate of the number of genes in Lilium was entirely arbitrary and he had no direct evidence for a correlation of "ultimate chromomeres" and genes. In salivary chromosomes of Drosophila, however, a great number of workers have proven beyond doubt that the visible "bands" are correlated with certain genes. A recent analysis of the salivary chromosomes of Sciara in collaboration with Dr. Helen Crouse (in press) has shown that the "granules" and "bands" are misinterpretations of a very complicated spiralization of a bundle of chromonemata. What has been described as a "chromomere" corresponding to a gene represents in reality a region of relatively considerable length along the chromonema. The cytogenetic work on Drosophila salivary chromosomes is not evidence for a "chromomeric" structure of the chromonema, but shows that certain sections of the uniform chromonematic thread correspond to definite genes and that the detailed nature of the coiling in these interphase chromosomes is closely correlated with a genetic specificity on a submicroscopic level.

In summary this is the evidence against the existence of "chromomeres": (a) In living cells the microdissection experiment of Chambers (1924) shows that the leptotene chromosome can be stretched into a uniform thread. (b) In several plants such as Tradescantia (Kaufmann, 1931; Swanson, 1943), Allium and Aloe (Koshy, 1934, 1937), Hyacinthus (Naithani, 1937), and in the grasshopper the leptotene chromosome consists of a uniform, coiled thread, Feulgen-positive throughout its length. No evidence of interchromomeric fibrils can be found. The leptotene coils can be followed into the pachytene where they increase in width and decrease in number. This explains the observation of many authors (e.g., Belling, 1931) that the "chromomeres" increase in size and decrease in number during the course of prophase. (c) In the diplotene chromosomes of the grasshopper no "chromomeres" can be seen in the large loops of the chromatids. If specific "chromomeric" granules were present at leptotene they should be visible also in the chromonema of diplotene. (d) McClintock (1944) has shown in maize that at least one gene is located in the interchromomeric thread between the terminal knob and the first "chromomere" on chromosome nine. This disproves definitely the idea, at least for maize, that the genes are necessarily located in the "chromomeres" which are connected by non-genic fibrils.

*Diplotene chromosomes and "lamp-brush chromosomes"*

The coiling cycle in the grasshopper appears to be identical with that described by Swanson (1942, 1943) for Tradescantia. The leptotene coil develops into the major coil of diakinesis and metaphase through despiralization. There is no definite minor coil, but from late pachytene on, an irregular waviness appears on the loops of the chromatids, resembling an incipient helix. A minor coil was seen in spermatocytes of another orthopteran, Podisma, by Makino (1936). In Trillium (Huskins, 1941) there seems to be a similar waviness instead of a definite helix as was demonstrated for Tradescantia. This difference in the appearance of the minor coil seems to be mainly one of timing of the spiralization cycle as Kuwada (1938) has suggested. In the grasshopper the chromatids have never been seen completely separated in diakinesis or metaphase. Their coils sometimes appear

interlocked as Kuwada (1938) found in Tradescantia, but this could not be definitely determined. Swanson (1942b) has shown that the terminalization of chiasmata is correlated with the despiralization of the major coil in Tradescantia. The same process takes place in the grasshopper and it is most likely that here, too, terminalization of chiasmata is the consequence of despiralization of the major coil.

The diffuse appearance of orthopteran as well as most other animal chromosomes in diplotene has made their analysis rather difficult. The chromonema is generally of smaller diameter than in plant chromosomes and therefore the delicate loops of the major coils escaped observation. This diffuse structure is due to a lateral separation of the chromatids in contrast to the usual appression of the chromatids in plant chromosomes. Under certain conditions, and especially in diakinesis, plant chromosomes also show a separation of chromatids. They then give the same pictures as diplotene chromosomes of animals (see the anaphase chromosome of desynaptic Trillium in Figure 9 of Sparrow, Huskins and Wilson, 1941; Swanson, 1942a, 1943, and Kuwada and Nakamura, 1938 for Tradescantia). Plant and animal chromosomes have often been described as reticulate or vacuolated. Grégoire and his school based on this their "vacuolization hypothesis" of chromosome structure. All their pictures can today be explained on the simple assumption of a multiple stranded helix with the chromonemata more or less appressed or opened up.

When the lateral separation of the chromonemata is great and the loops only faintly stained, the chromosome may appear covered with a layer of achromatic material (often described as "matrix" or "sheath"; see for instance Lee, 1921 and McClung, 1941, Figure 7). Probably many a "matrix" in the literature is nothing but the apparant connection between faintly staining outer loops, running at an even distance from the darker core of the chromosome where the chromonemata overlap. Makino (1936) published some photographs of diakinesis and metaphase chromosomes of Podisma which at first seem to contradict my interpretation of these stages. He shows a dark inner coil sometimes appearing double, surrounded by a light "matrix." Faint strands are sometimes seen to connect the central spiral with the border of the "matrix." Yet it is very easy to understand these figures with the help of a model of four wires coiled together. When two are maximally separated laterally and two stay appressed in the center, Makino's coil and matrix become explainable. The outer coils are not at all or only faintly stained in his gentian violet preparations and their outer boundary suggests the presence of a "matrix."

The previous studies of diplotene chromosomes of Orthoptera have completely ignored these outer gyres of the chromonemata. They were described as woolly threads or brushlike projections on the surface of the chromosome, but not as an essential part of it. Thus the pictures of Hearne and Huskins (1934), Nebel and Ruttle (1937), Darlington (1936), and the McClung school are based on optical illusions or too light staining. What were described as "chromomeres" in this stage are the points of overlap of the chromonemata. Darlington (1936) has studied relational coiling of chromatids and chromosomes in pachytene and diplotene. What he pictured as one single chromatid, however, is not a continuous structure, but a series of nodes of separate overlapping major coils. His relational coil of chromatids is therefore an optical illusion. Only a complete stretching of

the major coil could reveal whether the chromatids are wound around each other (see Kuwada, 1938).

Many oöcytes and spermatocytes in diplotene undergo a so-called "diffuse stage," which is correlated with the growth of the cell. The chromosomes stain only faintly and lose their definite shapes; they may even disappear into a reticular structure. In the grasshopper the diffuse nature of the chromosomes is due to the loosening and separation of the individual chromonemata of the major coil. This more or less pronounced loosening up of the gyres, combined possibly with some chemical changes in the composition of the chromatin, can explain the appearance of diplotene chromosomes during this stage in spermatocytes and oöcytes.

The diplotene chromosomes in the large oöcytes of some vertebrates have particularly interested the cytologist ever since their discovery by Rückert in 1892, because of their tremendous size. Their fuzzy and brush-like appearance warranted the name "lamp-brush chromosomes." Duryee (1937, 1938, 1939, 1941) has recently studied these chromosomes in great detail in the frog and salamander, and concludes that (1) they represent paired gelatinous cylinders in which the chromomeres are embedded. (2) From these chromomeres lateral loops grow out. He likens this growth to that of a crystal or the reproduction of a virus. (3) In a later stage, before the maturation divisions take place, these lateral loops are thrown off into the cytoplasm as genic products essential for the early embryo.

Painter (1940) came to somewhat different conclusions. He considers "lamp-brush chromosomes" to be chromosome aggregates, which originated through endomitosis and the loops to correspond to whole chromosomes. Material from thousands of such chromosomes, he maintains, is thrown into the cytoplasm as substrate for the synthesis of cleavage chromosomes. Koltzoff (1938) thinks that the lateral projections are side branches of the chromomeres which then are given off into the cytoplasm.

In contrast to Duryee, Koltzoff, and Painter, it is here suggested that "lamp-brush chromosomes" are typical diplotene chromosomes which differ from other diplotene chromosomes only in the tremendous longitudinal growth of the chromonemata. The loops are then the major coils of the laterally separated chromonemata, the "chromomeres" are simply overlaps of the strands just as in diplotene chromosomes of the grasshopper. Figure 12 shows a fragment of a "lamp-brush chromosome" of a frog oöcyte, smeared in aceto-orcein. The somewhat distorted large loops of the major coil and the minor coil are easily visible.

The evidence for this interpretation may be summarized as follows: (a) The loops are continuous as Rückert (1892) has already observed. He followed the chromonema for several turns. He also pointed out that the granules ("chromomeres") are not real, but optical sections of the overlapping threads. The denser inner region of the chromosome he described as due to the radial arrangement of the threads. (b) "Lamp-brush chromosomes" are diplotene chromosomes and except for their greater size have the same appearance as the diplotene chromosome of the grasshopper. Since it has been shown here that the loops are simply the gyres of the major coil of the separate chromonemata, one can conclude that the corresponding appearance of the "lamp-brush chromosome" is the result of a similar structure. (c) Koltzoff (1938) has published drawings of cross sections of "lamp-brush chromosomes" (his Figure 10, b and c). These cross sections look like a star with characteristically eight rays. These eight rays are most likely the eight

half-chromatids which form independent loops, though Koltzoff saw them as brush-like projections.

The reduction in chromosome size just before the meiotic divisions is accomplished then not by throwing off parts of the chromosome or entire chromosomes, but by elimination of material on a submicroscopic level.

*The microscopic organization of chromosomes*

Kuwada (1939) in his review of chromosome structure predicted that the spiral theory might well prove capable of harmonizing the various hypotheses of chromosome structure. Such a uniform interpretation of the structure of all types of chromosomes is now possible. The unit of the chromosome is the chromonema, a microscopically uniform thread. This chromonema is never completely straightened out, but always shows some degree of spiralization. This coiling is not at random, but, as the salivary chromosomes and heterochromatic regions show, is an expression of the longitudinal differentiation of the chromonema and closely correlated with the genes. It is, in other words, an expression of submicroscopic structure and possibly the functional state of the gene (cf. heterochromatin). The microscopic uniformity of course does not exclude a great variability of submicroscopic structure and chemical composition along the chromonema. During the mitotic cycle there develops a condensed chromosome through despiralization of the incipient coil of early prophase. The differentiation of mitotic chromosomes, primary and secondary constrictions, satellites, and heterochromatic regions are expressions of the differential coiling of the chromonemata. In the resting nucleus of different tissues we often find different patterns of heterochromatin. It may be that differential spiralization of the chromonemata in resting cells is correlated with cell differentiation. The chromonema is not uniform in length, but it can vary greatly from cell to cell in the same organism, as well as in the same cell in different metabolic states. In many synthetically very active cells as for instance some oöcytes, nurse cells, gland cells (dipteran salivary glands), the total amount of chromatin is greatly increased. This is accomplished by an increase in the number of chromosomes (endomitosis, cf. Geitler, 1941), by a growth in length of the chromonemata (as in "lamp-brush chromosomes") or by both simultaneously (salivary chromosomes). In "lamp-brush" and salivary chromosomes the increase in length is tremendous and would be difficult to understand if only inert "genoplasm" or "matrix" (Koltzoff, 1938) had increased. More likely it is an increase in the volume of the gene complex, related to the greater metabolic activity. We have to look at the gene, therefore, not as a unit of constant and specific size as expressed in the "chromomere" hypothesis, but as a complex that is greatly variable in mass, depending on the metabolic activity of the nucleus.

## Summary

1. "Chromomeres" do not exist as definite structures. What has been described as "chromomeres" are (a) misinterpretations of gyres of the chromonematic helix (leptotene, somatic prophase); (b) points of overlap of chromonemata (diplotene); (c) heterochromatic sections consisting of more tightly coiled regions of the chromonema. The fundamental unit of the chromosome is a microscopically

uniform thread. The longitudinal differentiation of the chromosome is due to differential coiling of this chromonema.

2. "Lamp-brush chromosomes" are typical diplotene chromosomes, but with tremendously enlongated chromonemata. The side branches are the gyres of the major coils of the individual chromonemata, which have laterally separated from each other.

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